

**SALINITY STRESS INDUCED BIOCHEMICAL RESPONSES IN  
STYLOSANTHES SPECIES**

**THESIS  
SUBMITTED TO THE  
BUNDELKHAND UNIVERSITY, JHANSI (U.P.)  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

**in  
BIOCHEMISTRY**

**BY**

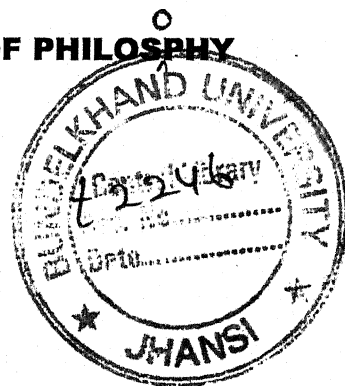
**SANCHITA TEWARI**

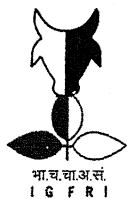
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# भारतीय चरागाह एवं चारा अनुसंधान संस्थान

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**Sub: Submission of Ph.D. thesis**

Sir,

I am forwarding herewith the thesis entitled "SALT STRESS INDUCED BIOCHEMICAL RESPONSES in *Stylosanthes* SPECIES" by Ms. Sanchita Tewari for the degree of Doctor of Philosophy in Biochemistry, Bundelkhand University, Jhansi. The work has been carried out at Indian Grassland and Fodder Research Institute, Jhansi under the supervision of Dr. Amaresh Chandra.

Thanking you,

Yours faithfully

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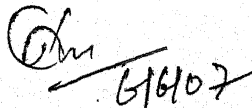
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## Certificate

It is certified that the thesis entitled "Salt stress induced biochemical responses in *Stylosanthes* species" is an original piece of work done by Sanchita Tewari, M. Sc. under my supervision and guidance for the degree of Doctor of Philosophy in Biochemistry, Bundelkhand University, Jhansi.

I, further certify that:

- It embodies the original work of candidate herself.
- It is up to the required standard both in respect to its contents and literary presentation for being referred to the examiners.
- The candidate has worked under me for the required period at the Indian Grassland and Fodder Research Institute, Jhansi.
- The candidate has put in the required attendance and worked under me at Indian Grassland and Fodder Research Institute, Jhansi.
- No part of the thesis has been submitted for any other degree or diploma.

  
**Amaresh Chandra**  
(Supervisor)

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*Sanchita Tewari*

(SANCHITA TEWARI)

*List of abbreviations used in the text and their expansion:*

ACP	Acid Phosphatase
EC	Electrical Conductivity
GO	Glycolate Oxidase
PMS	Phenazine Methosulphate
EST	Esterase
CAT	Catalase
NAD	Nicotinamide Adenine Dinucleotide
PAGE	Poly Acrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
MDA	Malondialdehyde
PRX	Peroxidase
PPO	Polyphenol oxidase
ADH	Alcohol Dehydrogenase
CAT	Catalase
RAPD	Random Amplified Polymorphic DNA
NBT	Nitro Blue Tetrazolium Chloride
APS	Ammonium Persulphate
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
EST	Expressed Sequence Tags
UPGMA	Un-weighted Pair Group Average Method with Arithmetic Mean
GOT	Glucose oxaloacetate transaminase
LAP	Leucine amino peptidase
GR	Glutathione reductase
GDH	Glutamate Dehydrogenase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
MDH	Malate Dehydrogenase
FBA	Fructose Biose Aldolase
PGM	Phosphor Gluco Mutase
SAHN	Sequential Agglomerative and Hierarchical & Nested
SDS	Sodium Dodecyl Sulphate
NADP	Nicotinamide Adenine Dinucleotide Phosphate
PVP	Polyvinylpyrrolidone
TBE	Tris Borate EDTA
AP	Ascorbate Peroxidase
IDH	Isocitrate dehydrogenase
GB	Glycine Betaine
GR	Glutathione Reductase
ROS	Reactive Oxygen Species
OA	Osmotic Adjustment
TAE	Tris Acetate EDTA
RM	Relative Mobility
STS	Sequence tagged sites

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## INTRODUCTION

The crops provide over 90% of the human need for calories. The cereals provide roughly 75% of the world's plant derived calories; root crops, oilseeds and the rest of the world's caloric needs are provided by vegetables and forage feed animals. However, marine sources provide only 3% of the calories. The world crop production is limited largely by environmental stresses. Statistics and their sources vary to a large extent but all point to the same conclusion (Blum, 1988). For instance, Dudal (1976) estimated that only 10% of the world's arable land may be categorized as free of stresses. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982; Bray *et al.*, 2000). Salinity is becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050. However, the largest increase in acreage of saline habitat is occurring through salinization of agricultural fields (San Pietro, 1982). The salt concentration increases in soils that experience long term irrigation and poor drainage, particularly in arid regions. Agricultural management plans that involve repeated cycles of water use and lying fallow in the summer further increase soil salinity. Although these programs result in increased salinity and dramatically reduce crop productivity and yield (Greenway and Munns, 1980; Ashraf, 1994), they are necessary due to the marginal nature of the land in use. The problem of salinity in agriculture will only increase as populations increase and larger tracts of presently unused or marginally used lands are brought into service. The countries most affected by agronomic salinization are often those that have a critical need for agricultural products and relatively small proportion of arable land. Salinity is widespread and is estimated to affect 10% of the world's land surface (Richards, 1995). The worst affected regions are in the semi-arid zones where population increase is greatest and where food production is most vulnerable because of problems with over grazing, over cultivation and the large seasonal variability in rainfall. Salinity usually occurs in conjugation with other soil problems. It is often associated with sodicity and high pH and hence with problems of soil structure, water permeability and nutrient imbalances. Waterlogging can also be an associated problem. All of these factors impose constraints on plant growth and when drought occurs, as is likely in many salt-

# INDIA

## SALT AFFECTED SOILS

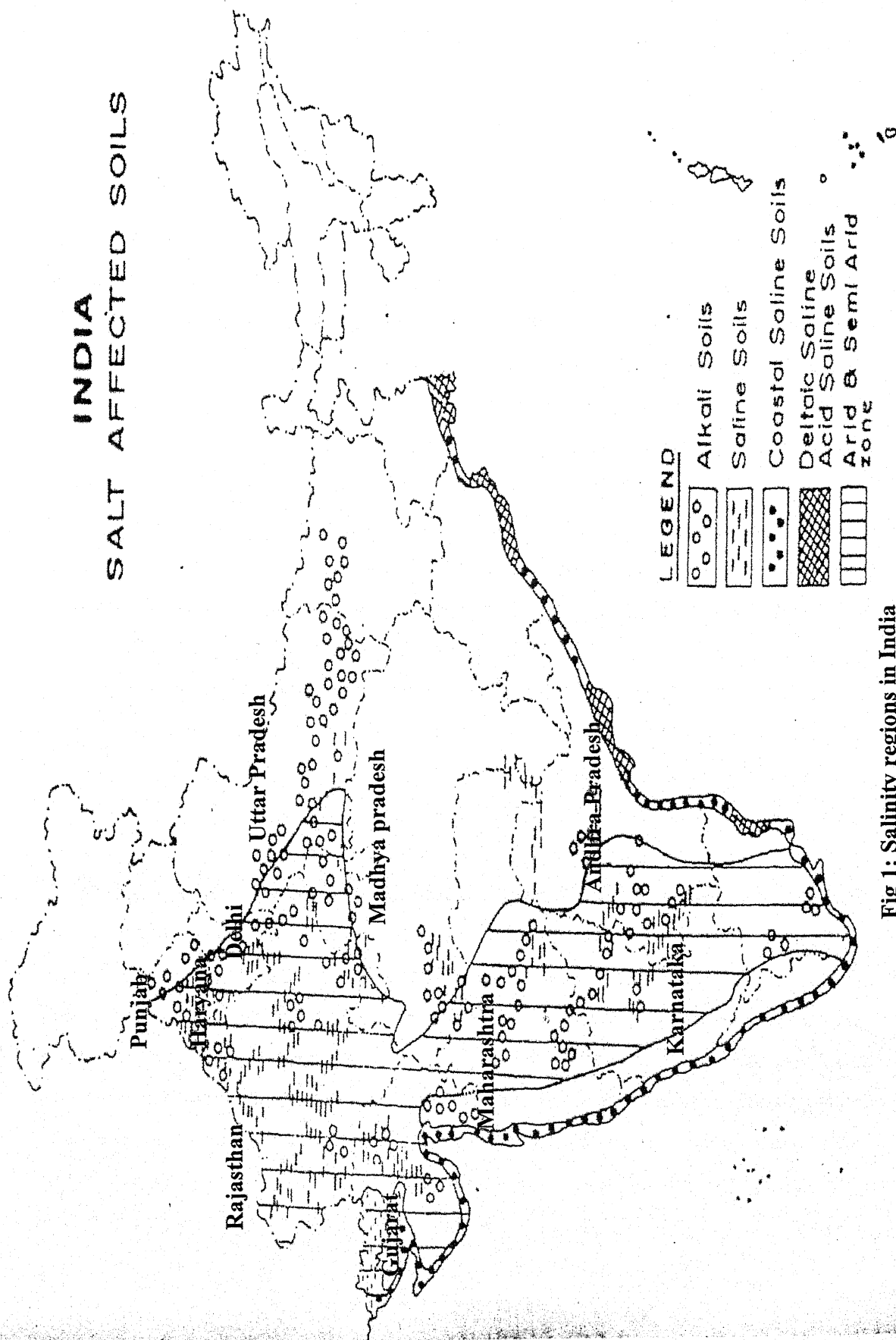


Fig 1: Salinity regions in India

affected regions, the root system of plants are often inadequate to cope with this additional stress. Low crop yield are, therefore, inevitable. Singh and Bandyopadhyay (1996) reported that salt affected soils occupy nearly  $2.5 \times 10^6$  ha in the Indian portion of the Indo-Gangetic Plains (IGP) and most of them are alkaline soils. Gupta and Gupta (1997) defined the areas of Indian soils affected by salinity and alkalinity (Fig. 1). Acreage of salt affected soils within the canal command suggested that introduction of irrigation hastens development of salinity problems through redistribution of salts inherently present in the soil surface. Salinity stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al.*, 2001). Drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and /or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano *et al.*, 1999; Zhu, 2001a). Oxidative stress, which frequently accompanies high temperature, salinity or drought stress, may cause denaturation of functional and structural similar cell signaling pathways (Shinozaki and Yamguchi-Shinozaki, 2000; Knight and Knight, 2001; Zhu, 2001b, 2002) and cellular responses, such as the production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes (Vierling and Kimpel, 1992; Zhu *et al.*, 1997; Cushman and Bohnert, 2000). Salinity can affect growth and yield of most crops, high salinity is known to cause hyperionic and hyperosmic effects in plants, leading to membrane disorganization increase in activated oxygen species production and metabolic toxicity (Hasegawa *et al.*, 2000). Salt marshes and mangrove swamps are characterized by sharp changes in species composition over short distances. On a physiological scale, adaptations to salt tolerance are similar to those for cold, oxidative, and water stress tolerance (Bohnert and Jensen, 1996). Several proteins induced by water stress and chilling and the signal transduction pathways may be very similar for these three stressors (Wu *et al.*, 1997). There is a rich complexity of environmental heterogeneity in saline environments. The ions involved in creating saline conditions can be diverse (sodium, calcium, potassium, sulfate, chloride, carbonate, etc.) and may contain toxic levels of boron and selenium. The combined effects of these ions can induce synergistic or antagonistic responses in plants. Along with the high ion

concentration, plants may experience limitations in nutrient availability, mechanical damage, and flooding in saline ecosystems. In addition there is wide spatial and temporal variation in salinity.

Due to the complexity of environmental stresses in saline habitats, adaptations are also complex and diverse. The complexity of adaptation to saline environments and its interaction with other important abiotic stressors provides a worthy and timely research topic in plant stress physiology. Through evolutionary time, many indigenous plants from a wide variety of families have adapted to salt rich regions. However, same cannot be implicated for most agricultural species. Sacher and Staples (1984) reported that the integrated biological and physical processes associated with saline land agriculture need to be understood in order to improve crop yield without amplifying the salinization problem. The development of new cultivars or the selection of new species with salt tolerance is important for solving this problem. Therefore, understanding the mechanisms by which native plants have adapted to saline environments, discovering the genes that determine those mechanisms, and transferring the genetic traits into agricultural crops are goals of many research laboratories around the world. Therefore, studying the physiology of salt tolerance will shed light on a number of different aspects of plant stress physiology and may help to develop a unified model of stress tolerance in plants. The complex plant response to abiotic stress, include up and down regulation of many genes through a well organized cascades, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects, or temperature, membrane fluidity changes) trigger the downstream signaling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signaling and gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death. The ongoing elucidation of the molecular control mechanisms of abiotic stress tolerance, which may result in the use of molecular tools for engineering more tolerant plants, is based on the expression of specific stress related genes. These genes include three major categories: (i) those that are involved in the signaling cascades and in

transcriptional control, such as MyC, MAP kinases and SOS kinase (Shinozaki and Yamguchi-Shinozaki, 1997; Munnik *et al.*, 1999; Zhu, 2001b), phospholipases (Chapman, 1998; Frank *et al.*, 2000), and transcriptional factors such as HSF, and the CBF/DREB and ABF/ABAE families (Stochinger *et al.*, 1997; Schoffl *et al.*, 1998; Choi *et al.*, 2000; Shinozaki and Yamguchi-Shinozaki, 2000); (ii) those that function directly in the protection of membranes and proteins, such as heat shock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA) proteins (Vierling, 1991; Ingram and Bartels, 1996; Thomashow, 1998, 1999; Bray *et al.*, 2000), osmoprotectants, and free radical scavengers (Bohnert and Sheveleva, 1998); (iii) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters (Maurel, 1997; Serrano *et al.*, 1999; Tyerman *et al.*, 1999; Zimmermann and Senetac, 1999; Blumwald, 2000). The majority of studies on the physiological response of plants to stress are based on the assumption that the only possible way that a plant can survive under stressing conditions is to express preexisting genetic information that counteracts the effect of the stress. There is no difference between resistance (also called pre-adaptation), accommodation, acclimation and adaptation; in all cases the stress is considered as a trigger of the expression of a preexisting defense program. The kinetics of growth is therefore usually short, a few days to two weeks, and to avoid secondary effects, proteins and mRNAs are extracted soon after exposure to stress. To maintain growth and productivity, plants must adapt to stress conditions and exercise specific tolerance mechanisms. Plant modification for enhanced tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. In contrast to most monogenic traits of engineered resistance to pests and herbicides, the genetically complex responses to abiotic conditions are more difficult to control and engineer. Present engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. The current efforts to improve plant stress tolerance by gene transformation have resulted in important achievements; however, the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental

side effects, make this task extremely difficult. The halophyte *Mesembryanthemum crystallinum* has emerged as a model system for understanding the molecular response to salt stress. This plant switches from C<sub>3</sub> photosynthesis to crassulacean acid metabolism (CAM) in response to salt stress or drought stress. A large number of genes are concomitantly up- and down regulated for this switch to be operational (Bohnert *et al.*, 1995). More than a hundred genes are induced and probably transcripts three times that number are repressed in response to salt stress in *M. crystallinum*. Employing two dimensional protein gel electrophoresis, it has been noted that application of salt to plants brings about a major change in the protein profile (Ramgopal *et al.*, 1991). In organisms ranging from bacteria to higher plants, there is a strong correlation between increased cellular proline levels and the capacity to survive in effects of high environmental salinity. It may also serve as an organic nitrogen reserve that can be utilized during recovery. Today, we have a good idea of the diversity of physiological, morphological, and behavioral mechanisms used by plants to tolerate several different detrimental affects of salinity. Scientists are on the verge of understanding several mechanisms, at the gene regulation level, that determine salinity tolerance in halophytes. In particular, research with *Arabidopsis*, *Mesembryanthemum* (ice plant), and yeast have recently yielded significant breakthroughs. It is clear that there is no qualitative metabolic differences between halophytes and glycophytes (with the exception of salt glands) and that adaptation mainly concern quantitative differences in metabolic pathways related to ion homeostasis. The transduction pathways for osmotic and other environmental stress responses are likely to be very complicated and will involve number of signal molecules such as proline, protein, cyclic nucleotides, enzymes and inositol polyphosphates. Thus the precise mechanism (s) by which plants respond to high salinity remains unresolved. However at the molecular level, most of the changes are likely the result of alterations in the expressions of genes. Therefore, it is important to identify the relevant genes and characterization their regulation in response to salinity stress. Scientists have generally devoted their studies to the understanding of physiological effects in plants or reclamation of saline soils, but until recently not many scientists have given adequate attention to breeding salt tolerant crop plants. The reasons for less attention in this area could be that the salinity resistance involves a number of traits, including ability to resist

NaCl entry via membrane leakage or apoplastic pathway, preferential accumulation of  $\text{Na}^+$  in older leaves, and tolerance of NaCl within the leaf tissue. Salt resistant plant has to maintain growth in the presence of an osmotic stress and, concomitantly, avoid toxic concentration of salts in their cytoplasm. For the improvement of salt tolerance through selection and breeding the existence of genetic variation for tolerance in the gene pool of a crop species is of prime importance. Inter specific, intra-specific and intra-cultivar variation for tolerance provides scope for breeding and selection for its improvement. A consistent maintenance of degree of salt tolerance at different stages of life cycle of a crop is of vital importance for a rapid improvement of salt tolerance through selection, which can be effected at any growth stage (Ashraf, 1994). For instance in a number of studies conferment of degree of salt tolerance at different growth stages has been found in many crop species, e.g. linseed (Ashraf and Fatima, 1995), safflower (Ashraf and Fatima, 1995), sunflower (Ashraf and Tufail, 1994) and *Brassica juncea* (Ashraf *et al.*, 1994). In another study salt tolerance improved at the seedling stage in three forage legumes *Medicago sativa*, *Trifolium alexandrinum* and *Trifolium pretense* and was conferred at the adult stage (Ashraf *et al.*, 1986). Ashraf and Sharif (1998) experimented on *B. carinata* and concluded that salt tolerance varies with the change in stages of its life cycle. Thus selection based at one particular stage may not produce individuals tolerant at all growth stages. Salt toxicity is avoided by employing compatible solutes for osmotic cytoplasm adjustment and by confining salt, in particular  $\text{Na}^+$ , to the vacuole and also so to the apoplast. The leaves of such plants may also contain salt glands. These glands accumulate excess salt and subsequently excrete it. This excretion may be explained as a special case of turgor down regulation. Other plants that apparently lack the  $\text{Na}^+/\text{H}^+$  antiporter accumulate organic solutes and  $\text{K}^+$  salts; they prevent  $\text{Na}^+$  influx to the roots and its translocation to the more sensitive shoots. The latter is accomplished by selective  $\text{Na}^+$  absorption from the ascending xylem and its recirculation to the roots via the phloem. Sodium ions permeate into plant cells through outward rectified cation channels that apparently open in response to  $\text{Na}^+$  induced polarization. The presence of  $\text{Ca}^{2+}$  and  $\text{K}^+$  enhances  $\text{Na}^+$  exclusion by controlling channel selectivity. High  $\text{K}^+$  concentration in the medium also enhances its adequate supply to the plant in the presence of excess  $\text{Na}^+$ . Genetic studies revealed that salt tolerance is principally due to additive gene effects;

however, the underlying molecular mechanism for the salt tolerance has scarcely been investigated. In recent years, some of the salt induced proteins, which were produced by the salt tolerant species, have been identified by two dimensional electrophoresis (Moons *et al.*, 1997; Ramani *et al.*, 1997). More than 35 polypeptides have been detected in this category. Prominent rice proteins responsive to salinity stress are the SalT, Lea (Group II, III), HSP 100 family, and OSR 40 proteins analysis makes it easy to identify accumulated proteins at specific stages of salinity stress, but mechanisms of metabolic changes and genetic regulation under salinity conditions remains obscure. While the search for genotypes with required levels of salt tolerance has confirmed primarily on screening the available range of genotypes, however efforts have expanded in the last 30 years to develop new genotypes for improved salt tolerance, via selection and breeding, using agronomic selection criteria, such as yield and survival, but with limited success. Only a limited number of plant genes with a defined function have been identified cloned and characterized. However there is an immediate need to enrich the data base on novel stress responsive genes. These genes need to be isolated and cloned from as many as diverse source as possible. The range grasses and legumes which grow in extreme conditions may constitute some of the best systems to isolate such genes. The recent upsurge in activities concerned with identifying genes with unknown functions through research on ESTs (Expressed Sequence Tags) and sequencing of total genomes is a boon for stress work. However, genes identified, isolated and cloned by such approaches would need to be functionally characterized. Range grasses and legumes would be the most appropriate candidates to be looked for such gene (s) as they become adaptable to such environment since the time they have evolved.

In the past, mainly five species of *Stylosanthes* (*S. hamata*, *S. scabra*, *S. humilis*, *S. viscosa* and *S. guianensis*) have been introduced primarily from Australia and evaluated at different sites in India. This was in addition to the native perennial *S. fruticosa* Alston, which is widely distributed throughout the southern peninsular regions. Though the first record of *Stylosanthes* in transition dates back to early forties, regional evaluations of introduced germplasm in India started in the 1970s at various regional and main research centers depicted variability in plant height, number of main branches and

days of flowering. The genus *Stylosanthes* (Fabaceae) constitutes more than forty species (Kirkbide and De Kirkbide, 1985). Species of this genus are grouped into two subgeneric sections, sectt. *Styposanthes* and *Stylosanthes*, based on the presence of rudimentary secondary floral axis and two inner bracteoles in the former and no such axis and only one inner bracteole in the latter. Most species of *Stylosanthes* are diploid ( $2n = 20$ ) but polyploid species ( $2n = 40$  and  $2n = 60$ ) also exist. The latter are exclusively allo-tetraploid. Sectt. *Styposanthes* contains both diploid and polyploid species, while species in sectt. *Stylosanthes* are exclusively diploid. Five species namely *S. scabra*, *S. seabrana*, *S. hamata*, *S. guianensis* and *S. viscosa* are predominantly used as fodder legume in humid to semi-arid tropics of the country (Ramesh *et al.*, 1997; Chandra *et al.*, 2006). Due to its ability to restore soil fertility, improve soil physical properties and provide permanent vegetation cover it is playing a vital role in the development of wastelands in India. However, the most important limitation attached with this crop is the narrow genetic variability and low availability of diverse accessions. It is extensively utilized in pastoral, agropastoral and silvipastoral systems for animal production. It is also considered as nurse crop in plantation on degraded lands. Large gap in requirement and availability of forage seeds is one of the major constraints in development of fodder resources in the country. The importance of livestock in Indian agriculture is well recognized. However, low productivity livestock is a matter of concern, which is mainly due to the poor fodder and feed resources. With the increasing livestock population in India it becomes imperative to produce more forage biomass to meet the demand. There exist a critical shortage of fodder i.e. 80 million tones dry and 660 million tones green. To overcome this situation, there is need to increase fodder production by efficient utilization of all the land resources and technologies. Additionally, studying the genetic relationship of species showcase better options of relating the biochemical characteristics among the species. Variations detected by the molecular markers offers a number of advantages over morphological and biochemical as they are generally affected by environment and less representation of loci respectively. The power of PCR is derived from its ability to replicate large amounts of a specific section of DNA in a short period of time. At its limits, PCR is able to rapidly amplify a specific region of a single DNA molecule *in vitro* to yield sufficient quantities that can be cloned, sequenced, analyzed by

restriction mapping. In RAPD analysis single nucleotides of random sequence are used for amplification of DNA by PCR (Williams *et al.*, 1990). Polymorphism can be simply identified as the presence or absence of amplification products on agarose gels. Polymorphism generated by the RAPD assays have been used for the assessment of the variation (Williams *et al.*, 1990), fingerprinting (Chandra *et al.*, 2003), cultivars and pathotypes.

Although salinity is one of the important environmental stress, knowledge on salinity tolerance of different *Stylosanthes* species is scanty. Most of the changes caused by salinity stress are adaptive in nature which enable plants to survive in the stressed environment and are the result of changes in the polypeptide as well as in the activity of enzymes involved in synthesis of it and also the accumulation of many solutes. These are largely the result of altered gene expression. Study of such adaptive responses will lead to a correlation between the expression of specific genes and the degree of adaptation to various stresses. The expression levels of individual genes can be modulated in response to a variety of extracellular and intracellular signals. The complement of genes that are expressed within a cell at a particular time gives a 'snap-shot' of the proteins that it is currently producing.

Understanding the biochemical and molecular responses of salinity stress, therefore, requires the identification and quantification of proteins, intermediary metabolites and enzyme activities involved in several processes including osmolytes compounds which could be further involved in the stress adaptation process in the plants and finally the expression patterns of different stress responsive genes. The study shall be taken up with thirty seven lines of stylo comprising seven species and then the selected lines will be subjected for comprehensive biochemical analysis.

The proposed study has the following objectives:

1. Screening of *Stylosanthes* lines at different levels of salinity.

2. Monitor the biochemical changes including antioxidant activities in stress and un-stress plants.
3. Level of genetic relationship amongst the lines showing different responses towards salinity.
4. Expression analysis of stress responsive genes in selected lines doing RTPCR.

The study will lead to better understanding of the biochemical basis of salt stress tolerance in *Stylosanthes* species and also its relationship with generated isozyme patterns. This could be further utilized in understanding the mechanism involved in the stress adaptation process. As the study involves some of the important species of *Stylosanthes* performing better in different ecological zones the generated knowledge will further augment the use of suitable species for particular regions of the country. The study will also provide specific and reliable physiological and biochemical attributes which may help in screening of salinity stress responsive lines for its genetic improvement.

## REVIEW OF LITERATURE

### New insights into Taxonomy and Phylogeny

*Stylosanthes* is a genus of the subtribe Stylosanthinae, tribe Aeschynomeneae, subfamily Papilionoideae and family Leguminosae (Polhill and Raven, 1981). It is naturally distributed in tropical, subtropical and temperate regions of the Americas, in tropical Africa and South-east Asia. Classical taxonomic treatments of *Stylosanthes* have been mainly based on some aspects of the floral and fruit morphology (Mannetje, 1984). Mannetje (1984) reviewed the history of the genus *Stylosanthes*, which was established in 1788 and later critically revised by Vogel in 1838, Taubert in 1891 and Mohlenbrock in 1958 and 1963. In 1838 Vogel established (15 species) the main division of the genus into the sections *Styposanthes* and *Stylosanthes* (formerly *Eustylosanthes*), on the basis of the presence of a rudimentary secondary floral axis and two inner bracteole in the former and no such axis and only one inner bracteole in the latter (Kirkbide & De Kirkbide, 1985; Mannetje, 1984). The genus *Stylosanthes* has a monophyletic origin (Gillies and Abbott, 1994) and is closely related to *Arachis* (Lavin *et al.*, 2001), with the *S. guianensis* species complex as the most ancient group, clearly distinct from the rest of the genus. Liu *et al.* (1999) identified ten basal genomes, named A to J. Genome A1 appears to be the maternal donor to all tetraploid species with a known AABB genome (*S. scabra*, *S. sericeiceps*, *S. tuberculata*), while genome C is the maternal donor for all those with a known AACC genome (*S. hamata* (4n), *S. subsericea*, *S. sundaica*). Most species are diploid ( $2n=20$ ) but polyploid species  $2n=40, 60$  also exist in *Stylosanthes*. The latter are exclusively allotetraploid. Sect. *Styposanthes* contains both diploid and polyploid species, while species in sect. *Stylosanthes* are exclusively diploid. Stace and Cameron (1984) postulated that a tetraploid (4n) is a combination of a diploid (2n) species from sect. *Stylosanthes* and a diploid (2n) species from sect. *Styposanthes*. Low specificity shown by STS markers reduces its value when used for species identification. The conservation between *Stylosanthes* species seems to be close enough to allow most PCR primers designed for use in one species to be used in another. It is possible to search for

the genetic origin of polyploids in *Stylosanthes* by STS analysis and combining it with cytogenetic analysis.

### ***Stylo* in India:**

Over the past 45 years, many species of *Stylosanthes* have been introduced and evaluated at different sites in India. Since 1965, various species of *Stylosanthes* have been introduced from Australia, S. America, Africa and the USA. In Indian Grassland and Fodder Research Institute at Jhansi began evaluation of *Stylosanthes* germplasm in 1974 using accessions of *S. humilis*, *S. guianensis*, *S. sundaica* and *S. mucronata*. Of these, only *S. humilis* and *S. guianensis* were found to be promising (Magoon *et al.*, 1974). There has been a strong research effort at the IGFR on many aspects of *Stylosanthes* adaptation, germplasm evaluation, animal production, pathology and production systems. Evaluation in West Bengal showed *S. humilis* as the best species for marginal lands. Subsequent evaluation of *S. hamata*, *S. scabra*, *S. humilis* and *S. guianensis* as fodder in the states of Rajasthan (Mauria and Harsh, 1984), Andhra Pradesh, Maharashtra and Karnataka has identified *S. hamata* and *S. scabra* as superior species. By contrast, *S. guianensis* was the most suitable species for fodder production in Kerala and Manipur (Gupta *et al.*, 1989). In 1970, the Ministry of Rural Reconstruction identified 54 district and 18 contiguous areas as prone to drought and initiated a Drought Prone Area Program (DPAP) for integrated agricultural and pastoral development to stabilize the ecology and economy of over 60 M ha. Hyderabad (All India Coordinated Research for Dryland Agriculture started by the Indian Council of Agricultural Research, ICAR), Anantpur (Agriculture centers of the state of Andhra Pradesh), Ahmednagar (University of Maharashtra), Dhari (Grassland Research Station and Gujarat Agriculture University) and Jodhpur (CAZRI) were the major centers in this program. Of many species of *Stylosanthes* introduced in India since early seventies, the recently added species, *S. seabrana* shows great promise in terms of seed vigour, establishment as well as nutritional parameters. High regeneration potential further makes this species superior to *S. hamata* which have been naturalized in India. Molecular marker analysis indicated low level of polymorphism in available accessions of *S. seabrana*. However, it harbours good

seedling vigour and frost tolerance. Being one of the diploid progenitors, it provides an opportunity to artificially synthesize tetraploid *S. scabra*, using its pre-selected accession along with *S. viscosa*, a second progenitor. These artificial *S. scabra* genotypes could be directly used in breeding programmes.

***S. seabrana*:** Group of new *Stylosanthes* accessions, tentatively named *Stylosanthes* sp. aff. *S. scabra*, was identified at the early period of its identification. These accessions resemble *S. scabra* in many characters including growth habits, perenniality, and fruit shape with the last being one of the few key morphological characteristics used for classification at the species level in *Stylosanthes*. However, they differ from *S. scabra* in seedling vigor and flowering time. Further, unlike the widely used *S. scabra* cv. Seca, they are most tolerant, and well adapted to heavy soils of southern and central Queensland, Australia. It is perennial.

***S. scabra*:** Sub-shrub, erect to sub-erect to 1.5 m; stems with variable branching covered by short or long hairs with bristles. Leaflets oblong to oblanceolate, apex obtuse to mucronate, hairy on both surfaces. Stipules obovate, inflorescences obovate to elliptical, generally longer than wide, 10-30mm long. Loment with usually two fertile articles, the upper usually glabrous, sometimes pilose. Seed is small, yellow, 1.5-2.0 mm long. It is perennial.

***S. hamata*:** This species is reported from Brazil. The axis is sometimes difficult to find, but identification is normally unambiguous. Axis rudiment and two inner bracteoles present. It is annual and normally procumbent type.

***S. viscosa*:** Loments usually with two fertile articles. Seed yield is high. Ecotype variation is abundant. One group of ecotypes has a prostrate habit, spikes and leaves small, densely pubescent with short viscid hairs, stem much branched. Beak distinctly coiled and it is perennial.

*S. humilis*: Leaflets elliptic or lanceolate, inflorescences many times longer than wide. Loment may have two fertile articles. Always it is annual. The presence of bristles on leaflets can be variable, seeds usually black.

*S. guianensis*: Erect or suberect plant, leaflet lanceolate with acute apex, covered with different types of hairs. Inflorescence capitate, compound, many flowered. Loment generally with one fertile article.

*S. capitata*: Sub-shrub, prostrate or erect, to 1 m diameter or height, multibranched, leaflets elliptical-oblong sometimes obovate, apex acute or mucronate. Inflorescences oblong-ovate, longer than broad. Loment reticulated with 2 fertile articles. Axis rudiment is present.

#### **About salinity areas and their impact on growth and productivity:**

There was a great deal of variation among species and even cultivars in salt tolerance (Shannon, 1983) from sensitive ones that are prevented from normal growth by low concentrations of NaCl to the most resistant halophytes from saline habitats. Even among the halophytes, the variation is observed among the species (Dewey, 1960). The glycophytes, in general, grow well only under non-saline conditions. Germination of glycophytes may be inhibited by as low as 0.5% salt. The halophytes may germinate at salt stresses of 10 times that concentration. The limit for growth may be as low as 0.3% in some glycophytes. In contrast halophytes can grow on soil containing up to 20 % salt, although most grow on soils with 2-6% salt (Strogonov, 1964), and they can grow in solution culture with very high concentrations. The halophytes accumulate large amount of salt in their tissues. Some families tend to show either high or low limits of salt survival. The limit may be indicated by a cessation of growth or by an actual killing of the tissues in the form of necrosis or a marginal burn followed by a loss of turgor, falling of leaves and finally death of plants. Amzallag *et al.* (1990) showed that in Sorghum the adapted plant could develop and produce seeds under stress conditions that were lethal for the unadapted plant. All responses induced by the environment were not similar.

Adaptation of cultured plant cells from different species to several stress conditions showed that adaptation was a general response of plant cells. Adaptation could be induced rapidly if the stress was applied progressively. In fact, like cold acclimation, whole plants could also become salt tolerant by being exposed to lower non-lethal concentrations of salt. Research with glycophytic plant species has shown that upon exposure to high salinity, plants may exhibit a reduced growth rate, accelerated development and senescence or death if the stress was severe or prolonged as reported by Lazof and Bernstein (1999).

Like many other glycophytes, the sensitivity of *Arabidopsis* to salt stress was exhibited at all stages of development. For example, a brief (8 hr) treatment with 150mM NaCl during seed development stage resulted in callose deposition and abnormal changes in ovule and embryo structures indicative of cell death indicated by Sun and Hauser (2001). However in *Arabidopsis* salt sensitivity was most evident at the seed germination and seedling stages. Even after cold stratification of imbibed seeds to break dormancy, *Arabidopsis* seed germination was greatly impaired at salt concentrations at or above 75mM. After germination, seedling growth was also very sensitive to NaCl. Although lower concentrations of NaCl (<50mM NaCl) may have a slightly stimulating effect on fresh weight gain in culture media, higher than 50mM of NaCl clearly inhibits plant growth and eventually kills the plants. Normal tobacco cells could not grow in the presence of 100mM NaCl. After salt adaptation, some tobacco cells were able to tolerate five times that much salt reported by Hasegawa *et al.* (1994). Similarly alfalfa cells as well as intact plants have been adapted to tolerate very high levels of NaCl reported by Winicov (1991). To determine the potential of wheat grasses (*Elitrigia* Desv.=*Agropyron sensu lato*) as gene sources for the improvement of salt tolerance in wheat, six salt tolerant wheat accessions and 36 wheat grass accessions, representing 13 species were grown from seedling stage (Mc Guire and Dvorak, 1981). Plants were grown in gradually increasing concentrations of NaCl in hydroculture with one half strength Hoagland's solution. Tolerance to the NaCl was determined by scoring survival for the wheat on 250mM NaCl and for wheat grass by scoring survival in 500mM and 70mM NaCl. No wheat plants survived in 250mM NaCl. Several wheatgrass accessions had large

percentage of surviving individuals in 750mM NaCl. The four most tolerant species were *E. scirpea*, *E. pontica*, *E. junceiformis*, *E. diae*. Dvorak *et al.* (1985) reported that *E. pontica* is the most salt tolerant species. Although, these species are easily hybridized with wheat, their chromosomes normally do not pair with wheat chromosomes, which precludes introgression of *Elytrigia* genes into wheat chromosomes. The degree of genotypic variation for salt tolerance of the *Lolium* species (rye grass) and of the tall wheat grass *Elytrigia pontica* and saltmarsh grass (*Puccinellia ciliata*) was studied by Marcar (1987). All the species were shown to be relatively insensitive to NaCl upto 200mol m<sup>-3</sup> during germination, but higher concentrations were tolerated only by saltmarsh grass and Italian rye grass (*L. multiflorum*). Ten wheat and six barley genotype were tested for their response to soil salinity regimes varying from 0 to 16 m mhos/cm (Nair and Khulbe, 1990). Barley showed remarkable resistance to salt stress, linked to its capacity to resist efflux of potassium ions from the plant systems. Both crops showed substantial yield reduction at 12 m mhos/cm but barley still out yielded wheat by over 50%. There were significant interactions between salinity levels and genotypes in wheat but not in barley. Rice varieties, Pokkali, Getu and Nona Bokaro from India are the best sources of resistance to salinity. IR-4630 and IR-4763 showed high tolerance for salinity and IR-30, IR-32 and IR-36 had moderate salinity tolerance. IR-4227-28-3 and IR-4227-104-3 revealed outstanding tolerance for alkalinity and 52 advanced breeding lines showed moderate tolerance (IRRI, 1976). The salt ratings and relative grain yield (%) of the 18 cvs. of rice grown in saline soil in the Philippines were reported and it was concluded that cultivars with higher yield had low salt injury ratings. The whole plant growth and physiological responses to salt stress of the two sorghum species i.e., grain sorghum (*Sorghum bicolor*) and Johnson grass (*S. halepense*) were compared quantitatively (Yang *et al.*, 1990). Salt stress was induced by adding incremental levels of NaCl to a vermiculite medium until concentrations of 0.1M and 0.2 M were attained. Leaf number and leaf area reduction and dry weight reduction in the culm and leaves in response to salinity compared to controls were greater in *S. bicolor* than in *S. halepense*. Larger leaf growth reductions in response to salinity in *S. bicolor* were associated with higher tissue levels of Na<sup>+</sup> and Cl<sup>-</sup>. *S. halepense* had a lower Na/K ratio in the leaves as well as in the roots; the culm ratio was the same in both species. Extent of genetic

variation for salinity tolerance in the germplasm of pigeonpea (*Cajanus cajan*) and its wild relatives was studied by Subbarao *et al.* (1991). Among the genotypes of cultivated pigeonpea tested ICPL-227 was one of the most tolerant and HY3C was one of the most sensitive. None of the genotypes tested were able to survive beyond 30 days at 8dS/m or higher salinity levels. Among the wild relatives of pigeon pea, various species of *Athylosia* (now *cajanus*) *Rynchosia* and *Dunbaria* showed a wide range of variation in their salinity tolerance (critical levels from 4 to 12 dS/m). The salt tolerance of 761 alfalfa (*Medicago sativa*) accessions to NaCl during germination was studied. The accessions were characterized by sub-species, country of origin and centre of diversity (Rumbaugh and Pendery, 1990). The germplasm from the arid Indian and African centers excelled in NaCl tolerance during germination. Germplasm from the Falcata centre was least tolerant. *M. sativa* subsp. *sativa* was more than twice as tolerant as *M. sativa* subsp. *ambigua* or subsp. *falcata*. Germination studies on 20 cultivar of mungbean (*V. radiata*), 22 of blackgram (*V. mungo*) and two of cluster bean (*Cyamopsis tetragonoloba*) were conducted at five levels of salinity (3 to 18 m mhos/cm) 1/5 Hoagland nutrient solution in plastic containers (Maliwal and Paliwal, 1982). Germination of all the cultivars was delayed and decreased with an increase in salinity level. Seedling height was also significantly decreased with an increase in salinity level. Salt tolerance limit varied with the cultivar. Most of mungbean and blackgram varieties tolerate salt to the extent of 9 to 18 m mhos /cm. Both are salt tolerant at germination stage. However, clusterbean varieties are less tolerant to salinity than blackgram and mungbean. Some varieties of blackgram (Khargone-3, Urid 24-3, No. 48) and mungbean (S-72, H-45, No.525, Madira, RS-4) were more salt tolerant. Over 5000 accessions of hexaploid wheat were screened in solution culture salinized with sea salt (Kingsbury and Epstein, 1984). Screening of 85% seawater yielded 312 individual selections of capable of vigorous growth at germination and emergence. These were recovered and transferred to non-saline conditions for seed multiplication. Subsequent screening of the next generation was done over the entire life cycle at 50% sea water, resulting in the isolation of 29 salt resistant lines. Salt tolerant calli were selected from two *japonica* and two *indica* rice cultivars on basal media containing 6000, 9000, 12,000 and 15,000 ppm NaCl (Kim *et al.*, 1988). Frequency of callus formation decreased with the increase of NaCl in the medium, especially in India.

About half of the calli of japonica cultivars selected on NaCl amended media survived 12,000 ppm NaCl but none of the indica calli survived. In *Japonica* more plants were regenerated from calli selected on all concentrations of NaCl media than from NaCl free medium. Concentration of  $\text{Cl}^-$  in callus increased dramatically with increased NaCl content but peroxidase activity decreased. 48 lettuce (*Lactuca sativa*) cultivars were grown under non-saline (control, 1.0dS/m) and saline (9.3dS/m) conditions in sand cultures. Fresh leaf weights were compared on the basis of absolute and relative salt tolerance and mean yields (Shannon *et al.*, 1983). There was high variability in fresh weight in both control and saline environment, but generally variability in non saline environment was higher than in saline ones. The effect of NaCl on forage produced by plants regenerated from non-saline callus cultures was examined by Johnson and Smith, 1992. Weight of forage produced by rooted stem cuttings of regenerated plants was 33% higher at 50mM NaCl compared to cuttings of explant donor plants. Self progenies from four of five regenerants had higher progenies relative forage weight at 100mM NaCl than the original plants indicating increased NaCl tolerance.

#### **Dry matter and osmotic adjustment:**

The root growth is almost always less affected than shoot growth, so the root : shoot ratio increases. At low salinity, root growth may not decrease at all while shoot growth declines e.g. barley or it may even increase (e.g. Bermuda grass; *Cynodon* species; sorghum). These effects are clear even in the short term (one or two days) stress or before ions in the shoots would have build up to high levels. The influence of salinity on growth, water relations and photosynthesis in *Diplanchnie fusca* (L.) P. Baeuv. Salinity in the root environment was increased, the osmotic potential of the leaf sap and the difference between the leaf water potential increased progressively with each harvest. They also concluded that the primary effects of salinity on growth are not attributable to inhibition of leaf photosynthetic processes. On a mesophyll cell area basis, soluble protein was relatively constant in leaves developed at higher salinity level (100-400mM NaCl) while total chlorophyll decreased at all salinity levels. Plants produced less leaf area per unit dry weight as salinity increased, which may aid in water conservation. Shoot and root dry

weight, leaf production and leaf length were all substantially reduced in plants grown at high salinity. Total leaf area of plants at higher level of stress was only 20% of plants grown at low salinity. Both  $\text{Na}^+$  and  $\text{K}^+$  content of leaves and roots increased with salt concentration. Whilst the research for genotypes with required levels of salt tolerance has confined primarily on screening the available range of genotypes, however efforts have expanded in the last 30 years to develop new genotypes for improved salt tolerance, via selection and breeding, using agronomic selection criteria, such as yield and survival, but with limited success.

Although salt adaptation process did not receive as much attention as cold acclimation did, presumably because of technical difficulties in qualitatively implementing the 'salt acclimation' treatments. Nevertheless, the fact that adaptation can increase plant salt tolerance suggests that glycophytes do have salt tolerance machinery that may not be operating effectively in un-adapted conditions. Therefore, the difference in salt tolerance between glycophytes and halophytes appears to be quantitative rather than qualitative, and basic salt tolerance mechanisms are probably conserved in all plant species. The difference in salt sensitivity/tolerance may have resulted from differences in regulatory circuits or from gene alleles coding for key salt tolerance effectors. For example, the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene *AtNHX1* is not as highly inducible in *Arabidopsis* as its homologous gene in halophytes, and high level *AtNHX1* expression driven by the strong CaMV 35S promoter could significantly improve *Arabidopsis* salt tolerance (Apse *et al.*, 1999; Hamada *et al.*, 2001; Shi and Zhu, 2002). Sodium ions are not required for the growth of most land plants. Land plants do not seem to have transport systems specifically for  $\text{Na}^+$  uptake. However,  $\text{Na}^+$  can still enter plant cells via several routes. Since the concentration of  $\text{Na}^+$  in the soil solution is usually much higher than that in the cytosol of root cells.

For the improvement of salt tolerance through selection and breeding the existence of genetic variation for tolerance in the gene pool of a crop species is of prime importance interspecific, intraspecific and intra cultivar variation for tolerance provides scope for breeding and selection for its improvement (Ashraf *et al.*, 1986).

Salt tolerance in most crops varies during its ontogeny. For instance Akbar and Yabuno (1977) examined that different lines or strains of rice vary in their degree of salt tolerance at different stages of crop life cycle. Similarly, Kingsbury and Epstein (1984) working with different lines of spring wheat found that not only are there differences in salt tolerance among them but that individual lines also differ in their tolerance during various ontogenetic stages. Similarly results have been reported in pearl millet (Ashraf *et al.*, 1987) and alfalfa. Ashraf and Waheed (1993) reported that some salt tolerant lines of chickpea which were selected at the early growth stages showed consistent correlation between the degrees of salt tolerance at the early growth and adult stages.

A consistence maintenance of degree of salt tolerance at different stages of life cycle of a crop species is of vital importance for a rapid movement of salt tolerance through selection, which can be effected at any growth stage (Ashraf, 1994; Blum, 1988). For instance in a number of studies conferment of degree of salt tolerance at different growth stages has been found in many crop species, e.g. linseed (Ashraf and fatima, 1995), safflower (Ashraf and fatima, 1995), sunflower (Ashraf and Tufail, 1994) and *Brassica juncea* ( Ashraf *et al.*, 1994). In another study salt tolerance improved at the seedling stage in three forage legumes *Medicago sativa*, *Trifolium alexandrium* and *Trifolium pratense* and was conferred at the adult stage. Ashraf and Sharif (1998) experimented on *B. carinata* and concluded that salt tolerance varies with the change in stage of its life cycle. Thus selection based at one particular stage may not produce individuals tolerant at all growth stages.

### **Biochemical responses of the plants:**

#### **Proline:**

Abdel Hamid *et al.* (2003) examined the role of proline as apart of salt stress signaling in the desert plant *Panocratium maritium* L. The data showed that salt stress brought about a reduction of the growth and protein content, particularly at 300 mM NaCl that was significantly increased by exogenous proline. In the leaves salt stress up-regulated ubiquitin, a small protein targeting damaged proteins for degradation via the proteosome

up to five fold. Salt stress resulted in a decrease in the amount of ubiquitin-conjugates, particularly in the roots, and this effect was reversed by exogenous proline. Severe salt stress resulted in an inhibition of the antioxidative enzymes catalase and peroxidase activity, but the activity of these enzymes was maintained significantly in presence of proline. Mattioni *et al.* (1997) reported the alterations induced by water and salt stress in the proline metabolism and amino acid content of 5-day old seedlings of *Triticum durum* cv. Simeto. Most of the amino acids showed an increase with the induction of either of the stress, but proline increased more markedly than did other amino acids. They concluded that synthesis de novo is the predominant mechanism in proline accumulation in durum wheat. Use of a cDNA clone that encodes P5C-reductase from *Arabidopsis thaliana* showed no differences in the gene expression between controls and stressed plants, implying that the increase in enzyme activity is unrelated to the expression of this gene.

Compatible solutes that have been shown to accumulate under abiotic stress include sugar and sugar alcohols (polyols) as reported by Yancy *et al.* (1982), proline by Aspinall and Paleg (1981) and its analogues by Naidu *et al.* (1987) and a number of quaternary ammonium compounds (betaines) and tertiary sulfonium compounds studied by Wyn Jones and Storey (1981) and Rhodes and Hanson (1993). In *Lathyrus sativus*, a hardy grain legume which can withstand with stress, high proline accumulation was observed in leaves and roots under water stress (Tyagi, 1999). Although proline can be synthesized from either glutamate or ornithine, glutamate is the primary precursor in osmotically stressed cells.

Major contributions in stress molecular biology research by producing transgenic tobacco plants for enhanced cold and salt stress tolerance was produced. Following these two initial reports, transgenics showing tolerance to salt stress, water stress, oxidative stress, low temperature stress and high temperature stress have been produced employing various means. Tolerance to abiotic stresses so far has mainly been achieved through engineering for increased cellular levels of osmotically-active solutes (proline, glycinebetaine, mannitol, trehalose, fructans etc.). Another noteworthy point is that

increased levels of osmolytes have often enhanced tolerance for water stress, salt stress and cold stress at the same time, implying that genetic engineering by altering osmolytes is a fruitful approach for obtaining combined tolerance to different abiotic stresses. However, stress tolerance may not only be accounted for by osmotic adjustment. Transcripts corresponding to both cDNAs accumulate in response to NaCl treatment. Both these regulatory steps are keys to developing strategies for overproducing proline in a selected plant species. Binzel *et al.* (1988) found that tobacco cells adapted to 428mM NaCl could maintain cytosolic  $\text{Na}^+$  and  $\text{Cl}^-$  level at less than 100mM. Though mannitol only partially decreases the amount of inorganic ion accumulation in the cytosol, its protective effect as a compatible solute may be sufficient to give marginal growth advantage observed in transformed plants.

Only a limited number of plant genes with a defined function have been identified, cloned and characterized (Gibson and Somerville, 1993). However there is an immediate need to enrich the data base on novel stress responsive genes. These genes need to be isolated and cloned from as many diverse sources as possible. The range grasses and legumes which grow in extreme conditions may constitute some of the best systems to isolate such gene(s). The recent upsurge in activities concerned with identified genes with unknown functions through research on EST (Expressed sequence Tags) and sequencing of total genomes is a boon for stress work. However, genes identified, isolated and cloned by such approaches would need to be functionally characterized (Grover *et al.*, 1999). A classic example illustrating how physiology/biochemistry has been instrumental in unraveling novel genes for stress tolerance is the discovery of the role of the osmotic adjustment phenomenon in stress responses (Yancy *et al.*, 1982; Morgan, 1984). Range grasses and legumes would be the most appropriate candidates to be looked for such gene as they become adaptable to such environment since the time they have evolved. It should be pointed out that osmoprotection mechanism were not probably functional until severe stress occurs with the implication that osmotic adjustment may be critical to survival rather than to increase plant growth and crop yield under salt stress. However certain reports favor that osmotic adjustment can be related with yield parameters. Basnayake *et al.* (1995) investigated inheritance of osmotic

adjustment to water stress in sorghum. Populations of recombinant inbred lines were developed and characterized for osmotic adjustment for two of the crosses. These can be used to conduct experiments which test hypothesis about the contribution of the high-osmotic-adjustment genes to the grain yield of sorghum under a range of stress conditions.

Glycine-betaine is synthesized from choline in two steps, the first being catalyzed by choline mono-oxygenase leading to synthesis of betaine-aldehyde, which is further oxidized by betaine-aldehyde dehydrogenase. Salinity stress induces activity of both enzymes (Ramgopal, 1991). Genetic evidence that glycine-betaine improves salinity tolerance has been obtained from barley and maize (Grumet, 1986). There is also evident that degradation of proline in the mitochondria is directly coupled to respiratory electron transport system and ATP production. A pyrroline-5-carboxylate synthetase (P5CS) cDNA from moth-bean was introduced into rice. Expression of this P5CS transgene under the control of a stress-inducible promoter led to stress-induced overproduction of the P5CS enzyme and proline accumulation in transgenic rice plants. Second generation (RI) transgenic plants showed an increase in biomass under salt and water stress conditions. Levels of glycine-betaine in *Poaceae* species are correlated with salt tolerance highly tolerant *Spartina* and *Distichlis* accumulated the highest levels, moderately tolerant species accumulate intermediate levels and sensitive species accumulate low levels or no glycine-betaine.

### **Genetic relationship studies:**

#### **RAPD and STS analysis:**

Kazan *et al.* (1993) used 20 cultivars and accessions representing four agronomically important species of *Stylosanthes*, *S. scabra*, *S. hamata*, *S. guianensis*, and *S. humilis* for species relationships using RAPD as a marker. Approximately 200 fragments generated by 22 primers of arbitrary sequence were used to access the level of DNA variations. Relatively low levels of polymorphism (0-16% of total bands in pairwise comparisons) were found within each species, while between the species higher level of polymorphism

(up to 46%) was found. Very few polymorphisms (0.2%) were detected between the individuals of the same cultivar or accession. Phenogram of relationships among species was constructed based on band sharing showed four main clusters corresponding to each species were readily distinguished on this phenogram. The allotetraploid species *S. hamata* and its putative diploid progenitor, *S. humilis* were more similar to each other than to *S. scabra* and *S. guianensis*. No variation in RAPD markers was found between the two commercial *S. hamata* cvs 'Verano' and 'Amiga'. Cultivar 'Oxley' in *S. guianensis* was considerably different from the other cultivars and accessions of this species. The phylogenetic distinctions obtained with RAPDs were in agreement with other studies from morphology, cytology and enzyme electrophoresis. The low level of polymorphisms observed within each species suggested that interspecific crosses may be a better source for the construction of RAPD linkage maps.

Liu (1997) selected one hundred accessions of *S. scabra*, majority was collected from Brazil and most of the others came from either Colombia or Venezuela, to represent the geographical distribution of the *S. scabra* at Australian Tropical Forages Genetic Resource Centre, were analyzed using RAPD as markers. Seven of these accessions were found not to be *S. scabra*. Of the *S. scabra* accessions, the average dissimilarity value among Brazilian accessions (0.053) was much lower than that among Colombian (0.074) or Venezuelan (0.088) accessions, with an overall dissimilarity value of 0.059 among all the *S. scabra* accessions. Based on their dissimilarity values, most of these accessions could be separated into five species. Geographical distributions for most accessions in each of these groups were well defined. Limited long distance introductions/dispersions of *S. scabra* between these regions were detected and they were mainly confined to Brazilian genotypes. The clustering results based on RAPD were compared with those based on morphological-agronomical characters, and the groups produced by the two different methods did not always match.

Liu *et al.* (1999) tested STSs as genetic markers in *Stylosanthes*, 20 pairs of primers were generated out of which were randomly selected single-copy *Pst*I genomic clones, and the other five were from two known gene sequences. 24 genotypes representing 12 different *Stylosanthes* species analyzed with these primers. 13 of these primer pairs amplified

successfully, resulted a low level of genome specificity. This suggested low degree of genomic divergence within *Stylosanthes* species. In 312 entries (24 genotypes by 13 primer pairs), PCR were failed (little or no products) in only 16 cases. The number of banding patterns detected by each of these primer pairs varied from 2-12 with an average pair-wise polymorphism of 44.3%. The level of intraspecific variation detected on normal agarose gels was only 3.8%. Further evidence that diploid *S. hamata* and diploid *S. humilis* are progenitors of tetraploid *S. hamata* and that *S. viscosa* is a progenitors of *S. scabra*, was obtained.

Liu *et al.* (1997) identified that *Stylosanthes* sp. aff. *S. scabra* showed affinities with the allotetraploid species *S. scabra*, but distinct in a number of attributes. Several collections show potential as forage in northern Australia. 12 accessions have been analyzed using STS (sequence-tagged-sites) as genetic markers, and they all displayed STS phenotypes of typical diploid species. Taking into account their morphological similarities, the STS analysis provides strong evidence that *Stylosanthes* sp. aff. *S. scabra* might be a diploid progenitor of the allotetraploid *S. scabra*. This speculation was supported by cytological examinations. Somatic chromosome numbers of these accessions were counted and both were found to be diploid ( $2n=20$ ). The level of polymorphism among the 12 *Stylosanthes* sp. aff. *S. scabra* accessions, were estimated using RAPD as markers, was 7.8%, and the dissimilarity value between *Stylosanthes* sp. aff. *S. scabra* and *S. viscosa* (the other putative progenitor of *S. scabra*) was 89%.

Vander Stappen *et al.* (1999) designed 19 STS primer pairs on coding and non coding regions in nine published *Stylosanthes* genes, which were mostly derived from cDNA. Twenty STS markers were selected to determine genetic relationships among 63 genotypes representing 24 *Stylosanthes* species. A total of 148 alleles were amplified and analyzed, resulting in a genetic similarity value ranging from 0.62 to 0.98 among the species. Cluster analysis revealed three main groups and three subgroups, and most of the species were classified unambiguously. Allopoloid species were recognized by the occurrence of more than one allele per STS marker, indicating fixed heterozygosity. Sixteen STS markers were useful for the identification of genotypes within a species.

Inter-species relationships, as revealed by STS, were in general agreement with previous morphological and molecular relationship studies. These STS markers were useful as an additional tool for the identification of species, sub-species and genotypes in *Stylosanthes*, with a view to plant conservation and breeding. Curtis *et al.* (1995) analyzed RFLP, using peroxidase *O*-methyltransferase, phenylalanine ammonia-lyase and coniferyl alcohol dehydrogenase cDNAs isolated from *Stylosanthes humilis*, as probes, provided molecular evidence for the genetic origin of the naturally occurring allotetraploid genotype *Stylosanthes hamata* cv. Verano ( $2n=4x=40$ ). Hybridization patterns strongly suggest that the likely progenitors of *S. hamata* cv. Verano were a diploid *S. humilis* ( $2n=2x=20$ ) and a diploid *S. hamata* ( $2n=2x=20$ ). Genetic relationships between cultivated sunflower and selected wild sunflower species was also studied. Accessions used in the study in cultivated sunflower are CMS 234B, KBSH-1, Modern, PCSP1, RHA 229, PAC 1091, in interspecific hybrids are PCSP-1 X *H. praecox*, PCSP-1 X *H. argophyllus*, *H. argophyllus* X *H. praecox*, in diploid annuals are *H. praecox*, *H. argophyllus*, in diploid perennials are *H. grasseserratus*, *H. occidentalis*, *H. nuttali*, *H. Mollis*, in tetraploid perennials *H. strumosus*, *H. decapentalus*, in hexaploid perennials *H. tuberosus*. Using 40 random primers, 21 primers resulted in good amplification. The data structure included a total of 140 marker levels for 18 genotypes involving accessions of cultivated sunflower, wild sunflower species of different ploidy levels and a few interspecific hybrids. They observed that increase in genetic distance between the cultivated and wild sunflowers seems to reflect an associated decrease in genetic distance in crossability between them. Polymorphism between 19 soybean accessions listed as Wilkin (PI 548501), Dippes Fruhegelbe, Zefir (PI 556836), Jubilejnaja, semu 8107, fiskeby 5 (PI 360955), charkovskaja, vilnenis (PI 189867), wanda, kunitz (PI 542044), eszter, mlochovska (PI 423713), mandin kazon, belosnezka (PI 507674), Miriam, acme (PI 548498), clay (PI 548534), maple amber (PI 548592), Mc call (PI 548582). Only 22 of all the 40 random primers used in RAPD reactions showed polymorphism, 122 highly reproducible RAPD fragments were generated, 55 of them were polymorphic (46%) results in the selection of genetically distinct individuals. Study consisting on genetic diversity using 27 tomato cultivars namely Arka abha, Arka abhijit, Arka ahuti, Arka alok, Arka ashish, Arka meghali, Arka shreshtha, Arka vikas, Punjab chhuara, Punjab

kesri, Punjab tropic, selection-12, TH-802, Pusa 120, Pusa divya, Pusa gaurav, Pusa Hybrid 2, Pusa Hybrid 4, Pusa Ruby, Pusa Sheetal, Pusa Uphar , Best of all, Healani, Marglobe, Roma, Sioux, VFN-8 with the help of 42 random primers. Overall high levels of pairwise similarity and low levels of marker diversity implied the existence of limited genetic variation in the materials. Old introductions and locally developed cultivars of the 1970s exhibited significance greater genetic variation than the ones released during 1990s.

Study on 20 cultivars of Persian clovers namely Kermanshahi-1, Kermanshahi-2, Haftun, P513, Sechin, Haftchin, Nehavandi, Haftchin-Hamadani, Chegini, Doroud, Harati, Dehpir, Silakhor, Alashtar, Haftchin-Brujerd, Kasaroun, Bazneh, Elvijan, Reiham, Tajra, 8 primers out of 30 used produced repeatable bands. In total 83 bands were produced, out of which 66 bands were polymorphic. The least similarity belonged to Haftchin-Brujerd and Kazerun and the highest similarity belonged to Chegini and Haftchin-Hamadan. Study on elephant grass accessions namely Cameroon, Vruckwona, Napier, Mercker comum, Teresopolis, Mineiro, Mott, Pioneiro, CNPGL 27-5 and Roxo Anao (Dwarf purple). In total 160 DNA bands were generated with the use of 44 random primers and 23% of these bands were monomorphic. Cultivars Cameroon, Vruckwona were mostly divergent from others, with an average genetic distance of 0.34. Accessions having lowest average genetic distances from the others were Pioneiro and CNPGL 27-5, both with a distance of 0.25. Overall, genetic distances ranged 0.06 to 0.43, indicating little genetic variability, despite the contrasting morphology of the studied accessions. Afzal *et al.* (2004) studied genetic diversity of 21 mungbean accessions namely Barimung-2, Barimung-2, BU mug-1, BU mug-2, Pusa Baisakhi, CN 9-5, VC 6173A, VC 6153B-20-g, VC 6370A, VC 6173B-11, Basanti, KPS-2, SML 32, PDM 11, Barimung-3, Binamung-1, Binamung-3, Binamung-4, Binamung-5, Sonamung, Barisal Local as they were subjected to analysis using 34 decamer primers. Total 204 bands were generated with an average of 6.0 per primer and exhibited 75% polymorphism. This indicated a rather narrow genetic base of tested cultivars. Nayak *et al.* (2003) studied 12 taxa of bamboo namely *Bambusa vulgaris* Schrader ex Wendl, *Bambusa vulgaris* var. *striata* Schrad ex Wendl, *B. ventricosa* Maclure, *B. multiplex* var. *Silver stripe*, *B. multiplex*

(Lour.)Raeushel ex. Schult & Sehult.f, *Bambusa arundicea* Willd., *Bambusa balcooa* Roxb., *Dendrocalamus giganteus* Munro, *Dinocloa m'Clellandi* Kurz., *Cephalostachyum pergracil* Munro, *Dendrocalamus strictus* and *Sasa species* Makino and Shibata and were analysed using 30 random primers. A total of 137 polymorphic bands were amplified by using 10 selected primers. Results revealed that *B. multiplex* var. Silver stripe and *B. multiplex* were very closely related species and there was no variation with *B. ventricosa* another minor cluster was obtained between *Bambusa arundicea*, *Cephalostachyum pergracil* and *Bambusa balcooa*. Study on *Atriplex nummularia* Lindl, *Haloxylon recurvum*(Moq) Bunge ex Boiss, *Salsola baryosma*(R and S) Dandy and *Suaeda nudiflora*(Moq), after applying different salt treatments and examined their seed germination, total seedling length and vigor. After biometrical analysis *Haloxylon recurvum* showed highest seed germination (73.7%) in 4% salt solution followed by *Salsola baryosma* (62.3%), *Suaeda nudiflora* (59.7%) and *Atriplex nummularia* (45%). Seedling vigor index of *Haloxylon recurvum* was the highest (8.7%) followed by *Suaeda nudiflora* (4.2%), *Salsola baryosma* (3.15%) and *Atriplex nummularia* (0.708). *Haloxylon recurvum* perform best among all plant species.

An experiment conducted using barley (*Hordeum vulgare* L.) var BI-2 with 5 levels of saline irrigation water namely 0, 4, 8, 12, 16 dSm<sup>-1</sup> and 4 levels of presoaked seed treatment using 0, 500, 1000 and 1500 mgL<sup>-1</sup> of cycocel. Germination percentage, protein content, chlorophyll content, yield and yield attributes of barley decreased significantly with increase in salinity of water and markedly above the EC 8dSm<sup>-1</sup> while presoaking of seeds in cycocel progressively increased the germination percentage, chlorophyll, protein content, yield and yield attributes and maximum being at higher concentration, i.e., at 1500 mgL<sup>-1</sup>. Using the random amplified polymorphic DNA (RAPD) method, the genetic diversity of 19 greek landraces and 9 cvs. of durum wheat [*Triticum turgidum* L. var. *durum* (Desf.)] was studied. Two commercial bread wheat (*Triticum aestivum* L.) cultivars and one genotype of *Triticum monococcum* L. were also included in the study. 87 arbitrary primers (10-mer) were evaluated in a preliminary experiment and 15 of them were selected from the main experiments based on the quality and reliability of their amplification and the polymorphism they revealed. A total of 150

DNA bands were obtained, 125 (83.3%) of which were polymorphic. On average, 10 DNA bands were amplified per primer, 8.3 of which were polymorphic. The genetic similarity between all pairs of genotypes was evaluated using the Jaccard's or Nei and Li's coefficients; the values of the former ranged from 0.153 to 0.973 while those of the latter were slightly higher (0.265-0.986). Cluster analysis was conducted by the UPGMA and the Neighbor joining methods. Both methods broadly placed 26 durum genotypes into 1 branch while the other branch consisted of 2 subgroups. The *T. monococcum* cultivar stood apart from all other genotypes.

### **Enzyme activities:**

Sheoran (1979) studied amylase activity and isozymes of amylase on different parts of germinated mung bean [*Vigna radiata* (L.) Wilczek] cv. Pusa Baisakhi grown under treatment with NaCl, KCl, Na<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>SO<sub>4</sub>. These treatments resulted in the decrease in amylase activity in the cotyledons, whereas, in embryo axis, roots and leaves an increase was observed. The increase in activity was more under chloride salts than sulphate ones. The isozymic pattern of amylase remains the same in roots and leaves of the plants grown under different salts except that the appearance of one isozyme was delayed in leaves. However in embryo axis, the higher concentration of salts resulted in the decrease in number of isozymes.

Antioxidant components studied on comparatively in a salt tolerant (cv. Prasad) and salt sensitive (cv. Lepakshi) cultivar of foxtail millet (*Setaria italica* L.) under different NaCl concentrations. Under conditions of salt stress, the salt tolerant cultivar exhibited increased total superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity, whereas both enzyme activities decreased in acutely salt-stressed seedlings of the sensitive cultivar. At 200 mM NaCl, the tolerant foxtail millet cultivar responded with induction of cytosolic Cu/Zn-SOD and the Mn-SOD isoform at the protein level. The elevated cytosolic Cu/Zn-SOD and cytosolic APX activity correlates with an induced accumulation of their transcripts. Tolerant 5-day old seedlings grown during high salinity treatment (200 mM NaCl) contained a lower amount of Na<sup>+</sup> ions and showed a lower

electrolyte leakage than sensitive seedlings. Their study indicates that salt induced oxidative tolerance is conferred by an enhanced compartment-specific activity of the antioxidant enzymes in response to compartment-specific signals.

Dubey (1983) studied amylase, ribonuclease, protease and levels of substrates like starch, sugar and ribonucleic acid in endosperms of germinating rice seeds under different levels of salts. Lower concentration of NaCl caused increase in amylase activity but sharp decrease was observed with higher concentration and so was depletion of starch from endosperm. Levels of sucrose, reducing and total sugars decreased with salinity. Suppression of RNase activity was about 2 and 4 folds with 0.15 M and 0.3.MNaCl respectively. Salinity inhibited the rate of disappearance of protein from endosperm. Increased protease activity was noticed up to 48 hours followed by sharp decrease during late hours of germination where maximum inhibition of activity with higher salt concentration was observed under saline situation but significant decrease was noticed during later stages of germination.

Demiral *et al.*(2004) screened the effect of exogenously applied glycine betaine (GB) on the alleviation of damaging effects of NaCl treatment and studied the relative water content (RWC), MDA, and the activity of some antioxidant enzymes in two rice (*Oryza sativa* L.) cultivars differing in salt tolerance (salt tolerant-Pokkali and salt sensitive IR-28), comparatively. Leaf RWC of both cultivars under salt treatment showed an increase with GB application. The activities of superoxide dismutase (SOD), ascorbate peroxidase (AP), catalase (CAT), and glutathione reductase (GR) increased in levels of Pokkali, but peroxidase (POX) activity decreased under salinity. In IR-28, the activities of SOD, AP and POX increased, whereas CAT and GR decreased upon exposure to salt. When compared to the salt treated group alone, GB application decreased the activity of SOD, AP, CAT, and GR in Pokkali, whereas it increased the activity of CAT and AP in IR -28 under salinity. However, the activity of POX in IR-28 under salinity showed a decrease with GB application compared to the NaCl group. In addition, MDA of both cvs. under salt treatment showed a decrease with GB treatment.

A study included investigation of physiological parameters in marigold (*Calendula officinalis* L.) after long term salt induced oxidative stress under 0, 50 and 100 mM NaCl. Under high salinity decrease in total glutathione and an increase in total ascorbate (AsA+DHA), accompanied with enhanced glutathione reductase (GR, EC 1.6.4.2) and ascorbate peroxidase (APX, EC 1.11.1.11) activities were observed in leaves. Decrease in SOD (SOD, EC.1.15.1.1) and peroxidase (POX, EC.1.11.1.7) also induced by salinity. The decrease in dehydroascorbate reductase (DHAR, EC.1.8.5.1) and monodehydroascorbate reductase (MDHAR, EC 1.11.1.6) activities suggests that other mechanisms play a major role in the regeneration of reduced ascorbate. The changes in catalase (CAT, EC 1.11.1.6) activities, both in roots and in leaves, may be important in H<sub>2</sub>O<sub>2</sub> homeostasis.

### **Isozymes:**

Avila *et al.* (2003) assayed eighteen isozymes to characterized 147 accessions of *Vicia faba* L. from different origins. The most polymorphic isozyme systems were aspartate aminotransferase (AAT), fructokinase (FK), phosphogluconate dehydrogenase (PGD), peroxidase (PRX), and superoxide dismutase (SOD), which provided tool for characterization of collections. They also studied possible contamination of heterozygosity within certain inbred lines. This was the first time that fructose-biphosphate aldolase (FBA) system was described in *V. faba*. Among five improved varieties and one wild accession of lily of the valley (*Convallaria majalis* L.), using isozyme marker system the genetic distance and genetic diversity was estimated by Verron *et al.*, 1993. Five enzyme systems namely, esterase (EST), acid phosphatase (ACP), peroxidase (POD), phosphoglucomutase (PGM) and superoxide dismutase (SOD). Esterase system gave lowest polymorphisms, peroxidase and phosphoglucomutase systems gave highest polymorphism. Grandiflora of nowes, German and fortunea Lily of the valley appeared as genetically different the study indicated that certain genetic diversity exists among the types of lily of the valley the isozyme variation may be related to genetic variation. Isozyme banding patterns were studied for cultivars of lily by means of starch gel electrophoresis. An array of continuous histidine-citrate

buffer systems at eight ranges of pH and four extraction buffers were used. Using this technique catalase (CAT), esterase (EST), malate dehydrogenase (MDH), malic enzyme (ME), peroxidase (PRX), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI) and 6-phosphogluconate dehydrogenase (PGD) were assayed. Analysis indicated that the lily cvs. could be separated from other *Lilium* species, except for two L. x formonlogi cultivars: 'Hakuba' and 'hakuka' which could not be distinguished from each other by the isozyme patterns assayed here. This study showed that isozymes can provide useful biochemical markers for lily cultivar identification and to estimate the phylogenetic relationships among those cultivars. Vinthage and Chakraborty (1992) investigated 9 isozyme systems in cvs. and selected accessions of *Stylosanthes Scabra*, *S. guianensis*, *S. hamata*, using seeds collected from single plants. The banding patterns were used to discriminate among all cultivars of *S. scabra* and *S. guianensis* and the cv. Verano from the other cultivars and accessions of *S. hamata*. All accessions and cultivars of *S. scabra* and *S. guianensis* showed differences at a number of loci ranging from 1 to 6. The isozyme banding patterns obtained for the leucine amino peptidase-2 (LAP-2) and isocitrate dehydrogenase (IDH) were sufficient to place the cultivars into species groups. These differences were restricted to either one or two of the three isozyme systems, acid phosphatase (ACP), phospho-gluco isomerase (PGI-2) and LAP-2. In *S. hamata*, one seed lot of cv. Amiga and two seed lots of cv. Verano showed intra-seed lot differences in ACP. In *S. scabra*, one seed lot of cv. Cook showed differences in PGI-2 isozyme. This genetic variability was thought to have been caused by outcrossing. Genetic studies revealed that salt tolerance is principally due to additive gene effects; however, the underlying molecular mechanism for the salt tolerance has scarcely been investigated. In recent years, some of the salt induced proteins, which were produced by the salt tolerant species, have been identified by two dimensional electrophoresis (Moons *et al.*, 1997; Ramani *et al.*, 1997). More than 35 polypeptides have been detected in these categories. Prominent rice proteins responsive to salt stress are the Salt, Lea (Group II, III), HSP 100 family, and OSR 40 proteins analysis makes it easy to identify accumulated proteins at specific stages of salinity stress, but mechanisms of metabolic changes and genetic regulation under salinity conditions remain obscure. The genetic variability in the seed proteins and the enzyme alcohol dehydrogenase (ADH) in three representative species of

the genus *Cucurbita* was studied. The banding patterns were obtained by means of vertical block electrophoresis in polyacrylamide gel. A specific protein components and ADH isozymes were established in the polymorphic banding patterns which can be applied individually or in combination as potential biochemical markers for breeding purposes. The study provided by Stoilova *et al.* (2006), provide useful information on seed protein and ADH isozyme polymorphism of the most frequent used *Cucurbita* cultivars. The results may be applied as a potential biochemical marker for solving several genetic-breeding areas. Malaviya *et al.* (2005), reported inter and intraspecies variation in *Trifolium*. For zymogram pattern of five enzyme system was made to work out estimate of variability among 25 species representing 134 accessions. Peroxidase(POD), esterase (EST), superoxide dismutase (SOD), acid phosphatase (ACP) and glutamate oxalo transaminase (GOT) using starch gel, revealed 46 types of zymograms for Est isozyme pattern was observed which account to 4.38 estimate of variability. The rich variability present among these species can provide good source of gene transfer from wild to cultivated species which otherwise have no specific zymogram and exhibit low variability.

### **Chlorophyll content:**

Mohan *et al.* (2000) carried a experiment in which they estimate the chlorophyll stability index (CSI) of salt tolerance in rice using three salt-tolerant varieties (CSR10, TRY1 and CO43) and four salt-susceptible varieties (IR20, White Ponni [WP], ADT36 and Jaya) along with their hybrids in two soil types (normal and salt-affected soil). Under normal soil conditions, all varieties and their hybrids showed good levels of CSI. Salt tolerant cultivars CO43, TRY1 and CSR10 registered higher CSI values, whereas susceptible varieties WP, ADT36 and Jaya showed lower values under saline conditions, chlorophyll content was affected to a greater extent in susceptible varieties than in tolerant varieties. Hybrids IR20/CO43, ADT36/CO43, WP/CSR10 and IR20/CSR10, however performed better under saline conditions than under normal conditions. Hybrids were found to be more tolerant compared with their more susceptible parents, suggesting that salt tolerant is a dominant trait and it is possible to incorporate salt tolerance through hybridization

between tolerant and susceptible parents. Increased salt tolerance in hybrids is a consequence of increased seedling vigor.

### **Mechanism of tolerance:**

A study was conducted for the evaluation of important genotypes of *S. hamata* for forage yield at central research farm of IGFR JHANSI during 1981 to 1982. The systematic work on various aspects of the important pasture species of *Stylosanthes* done in India appears to be limited, though this pasture legume is reported to be common wild form in southern region of the country. It is generally recognized that *Stylosanthes* species are adapted to highly infertile soils, after resisting heavy grazing and water stress. Study on seed protein patterns of 182 *Stylosanthes* accessions, representing 16 species and two hybrids, by polyacrylamide gel electrophoresis of crude extracts. All species could be recognized by examination of photographs and densitometer traces of the gels. Within the species *capitata*, *guianensis*, *hamata* and *viscosa* considerable variation occurred, whilst less in *S. humilis* and *S. scabra*.

For further improvement of salt tolerance in a plant species it is a prerequisite to understand the mechanism of salt tolerance. Determination of pattern of ion uptake is crucial to unravel the mechanism of salt tolerance in a plant species. It is now evident that higher plants can tolerate high levels of salt either by salt exclusion or by salt inclusion (Flowers *et al.*, 1977; Greenway and Munns, 1980; Wyn Jones, 1981). For instance it was observed that *Trifolium alexandrinum* was more salt tolerant than *Trifolium pratense* (Winter and Lauchli, 1982) and this was due to its efficient exclusion of  $\text{Na}^+$  and  $\text{Cl}^-$  compared to later species. However, Croughan *et al.* (1978) and Ashraf *et al.* (1986) showed that a selected salt tolerant line of *Medicago sativa* accumulated higher  $\text{Cl}^-$  than the unselected. Salt resistant plants primarily maintain their growth by an increase of the amount of solutes in the cells and by subsequent turgor regulation. This mechanism may be supplemented by increase cell wall plasticity and decreased threshold turgor. The turgor decreased is sensed by a "turgor sensor" apparently in the plasma membrane. The sensor emits an "error signal" that is transduced to the activation of adaptive processes.

Salinity reduces the growth of glycophytes and many halophytes (Flower *et al.*, 1977) and through osmotic and specific ion effects. The primary responses of glycophytes to salinity are the restricted transport of salt to the shoots and the maintenance of a favourable water balance by the synthesis of organic solutes. With in particular glycophytic species, variation in salt tolerance might be expected to be associated with variation in one or both these basic responses. Comparative evaluation of the salt tolerance of the species has been numerous. Most of our agricultural species have been classified for salt tolerance. Comprehensive lists are available specifying threshold soil salinities for initial yield decline and subsequent rates of yield loss for each species. Intra-specific variation in salt tolerance has been reported widely. In some species, however, little or no variation between varieties has been reported. In such cases the lack of variation may be due to a narrowing of the genetic base of the species via domestication and selection for favorable environments, by which genes for adaptation to adverse conditions are lost or are at very low frequencies. In other cases, the lack of variation can be due to screening only a small number of the available germplasm. A little variation between six varieties of lettuce, while Shannon *et al.* (1983) screened 85 lettuce cultivars and breeding lines and identified significant variability in salt tolerance. For many glycophytes, but not all, differences in salt tolerance between varieties or lines have been closely associated with reduced uptake and accumulation of  $\text{Na}^+$  and /or  $\text{Cl}^-$  ions at the whole plant, shoot or leaf level (Lauchli and Wieneke, 1979). In such cases, improvement for tolerance might be facilitated by using ion exclusion from certain tissues as a broad physiological selection criterion. However, Lauchli (1984) found that where higher salt tolerance was not related to a higher degree of  $\text{Na}^+$  or  $\text{Cl}^-$  exclusion.

Legumes are either sensitive or moderately sensitive to salinity and are among the most important crop species. Lauchli (1984) noted that most legumes respond to salinity by salt exclusion from the shoots in many species including soybean (Wieneke and Lauchli, 1979), alfalfa and *Trifolium alexandrium*. The intra-varietals differences linked with  $\text{Na}^+$  and  $\text{Cl}^-$  exclusion have been reported for alfalfa and white clover, so in leguminous species, ion exclusion from certain tissues might be an effective selection criterion. This approach has been used in the woody species citrus and grapes. Although,

Cl<sup>-</sup> exclusion from soybean shoots was controlled by a single gene pair and Flowers and Yeo (1977) selected from high and low sodium transporting lines of rice, there is a lack of studies for non woody crops using Na<sup>+</sup> and Cl<sup>-</sup> exclusion as a selection criterion for improving increased salt tolerance.

A capacity to restrict shoots Cl<sup>-</sup> accumulation is no doubt only a broad physiological mechanism, but integrated result of many physiological characters. These include control of uptake at the root plasmalemma and tonoplasts of the cortex, phloem translocation and compartmentation in older leaves (Greenway and Munns, 1980). Nevertheless, shoot Cl<sup>-</sup> concentration is a tangible parameter that can be used for selection and breeding. When the specific physiological characters are identified and understood, and their genetic control determined, for then molecular biologists may attempt techniques such as a recombinant DNA to affect rapid advances in tolerance breeding.

Many environmental stresses including salt stress impair electron transport system which leads to the formation of activated oxygen such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub> and OH<sup>-</sup> (Smirnoff, 1993, Zhang and Kirkhan, 1996 and Chandra *et al.*, 1998). Superoxide dismutase (SOD) and ascorbate peroxidase along with the antioxidant ascorbic acid and glutathione act to prevent oxidative damage in plants (Allen, 1995). Therefore, the production of reactive oxygen species is the common consequences of various abiotic stresses, leading to the degradation of lipids, pigments, protein and DNA. To overcome this problem plants develop the antioxidant mechanism to minimize the damage. The magnitude of ROS production and antioxidant capacity decides the tolerance/susceptibility of the genotypes. Generally, tolerant genotypes showed significantly lower Na<sup>+</sup> and Na<sup>+</sup>/K<sup>+</sup> ratio in younger leaves; low lipid peroxidation, elevated levels of reduced ascorbic acid and increased activities of enzymes involved in the antioxidant system. Tolerant genotypes of rice also showed faster reduction in stomatal conductance and transpiration during the initial period of salt stress, whereas, susceptible lines showed a delay of 1 to 2 day before their gas exchange was significantly reduced. This helped to avoid high initial influx of salt with the onset of salinity and facilitate gradual acclimation. Tolerance to salinity

stress involves many of adaptive strategies including less  $\text{Na}^+$  uptake, greater portioning of  $\text{K}^+$  to younger tissue and  $\text{Na}^+$  to roots and older tissue, sensitive stomata and the up-regulation of antioxidant system.

### **Salt stress regulation of gene expression:**

Genes that are up-regulated by salt stress mainly belong to several groups, based on their possible functionality. These genes encode the LEA proteins, enzymes (involved in the biosynthesis of osmolytes, hormones, detoxification and general metabolism), transporters (ion transporters, ABC transporters and aquaporins) and regulatory molecules such as transcription factors, protein kinase and phosphatase. The most common and widely reported genes that are stress-regulated are perhaps the *LEAs* or *LEA*-like genes. *LEA* genes encode late embryogenesis abundant proteins (Baker *et al.*, 1988; Dure *et al.*, 1989). These genes are highly expressed in seeds during the desiccation stage following maturation, and in vegetative tissues in response to water deficit. They have been described in many plant species. Despite their wide occurrence, the functions of this group of polypeptides are ill defined except in a few cases where over expression of individual *LEA* genes resulted in some degree of stress protection. When plants are challenged with hyperosmolarity, accumulation of ions such as  $\text{Na}^+$  in the vacuoles can serve as a means to lower osmotic potential of the cells, and this process is perhaps cost-effective with regard to the amount of energy and resources spent. A related strategy used to lower the osmotic potential of the cell cytosol is to accumulate compatible osmolytes. For glycophytes, the capacities for  $\text{Na}^+$  compartmentalization and accumulation of osmolytes are both limited. Various compatible solutes such as proline, glycine betaine, and polyols can greatly reduce stress damage to plant cells. An increased production of osmolytes is a general phenomenon found in all plants in response to salt stress. This is clearly an adaptive strategy and transgenic plants with increased osmolyte production of reduced degradation (Nanjo *et al.*, 1999) showed improved salt tolerance. Besides reducing the osmotic potential of the cytosol to facilitate water uptake, many compatible osmolytes have additional functions such as protecting proteins from misfolding and alleviating the toxic effect of reactive oxygen species generated by salt

stress was reported by Smirnov and Cumber (1989) and Hong *et al.* (2000). Study on *Triticum* and *Aegilops* seedlings differing in their response to drought stress at the physiological and molecular levels. Modification in the expression level of five dehydrin (DHN) genes was also analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Five cDNAs coding for different DHNs were identified and characterized. These genes were not expressed in the well watered plants, but only in the stressed plants. Four of these cDNAs were related to novel DHN sequences. The results obtained clearly indicated a relation between the expression of these genes and tissue water content. In particular, in the resistant genotypes the expression of DHN genes was initiated even though tissue hydration levels were still high, indicating the involvement of these proteins in water retention in wheat also.

## MATERIALS and METHODS

### Materials

The experimental materials consisted of seven *Stylosanthes* species comprising 37 accessions procured from Indian Grassland and Fodder Research Institute (IGFRI) gene bank (Table 1, Fig. 2). All laboratory investigations were carried out at the Plant Physiology and Biochemistry unit of Crop Improvement Division, IGFRI. The healthy and mature seeds of all accessions were used to raise the seedlings in laboratory. Seedlings were raised fresh for each experiment in the germinator at 30°C or otherwise they were germinated straight way in the mixture of salts as well as in NaCl of different concentrations. The selected accessions were also germinated on the petri plates in the presence of different concentrations of NaCl. For RT-PCR analysis seedlings were raised in control condition without salt as well as after three days different concentration of NaCl was given and RNA was isolated at different time intervals. Interspecific and intraspecific genetic relatedness study was performed by isolating DNA with the help of buffer 'S' from the seedlings of each accession. Fresh and young leaves from well grown plants of five selected genotypes in triplicate were studied to investigate their performance for various physiological and biochemical attributes under control and salt stress condition imposed by providing once in a week Hoagland's nutrient media supplemented with NaCl. Before placing for germination, seeds were thoroughly scarified using sand paper for proper germination (Fig. 3). All laboratory experiments were carried out using sterilized petri plates.

### Chemicals:

The chemicals of different standard companies were used for the present investigation.

### Primers:

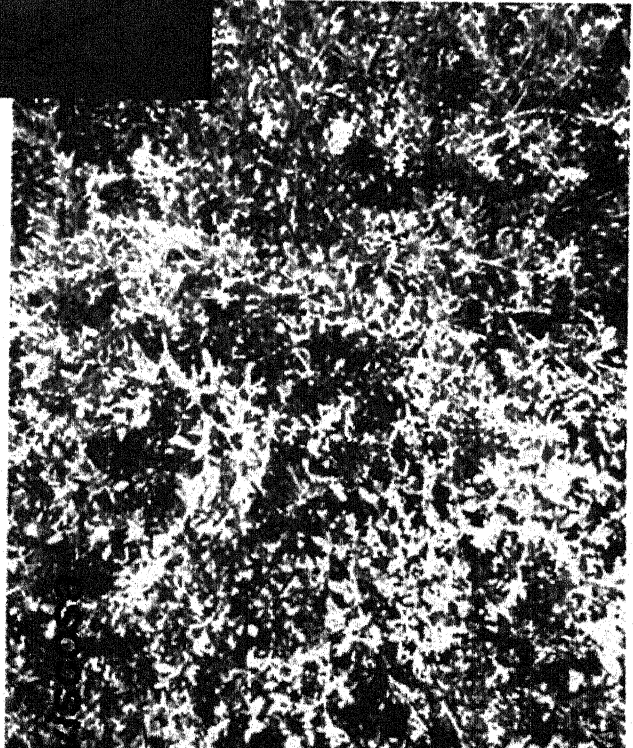
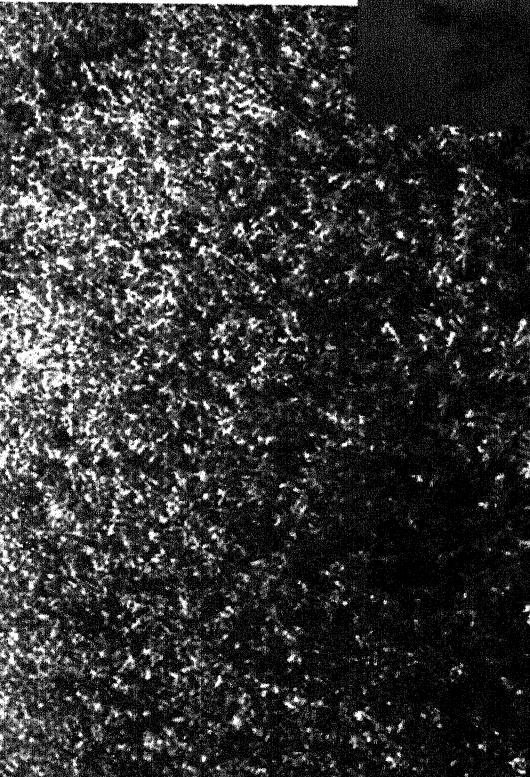
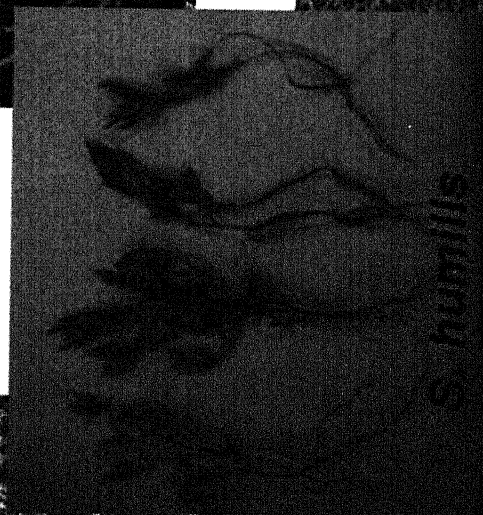
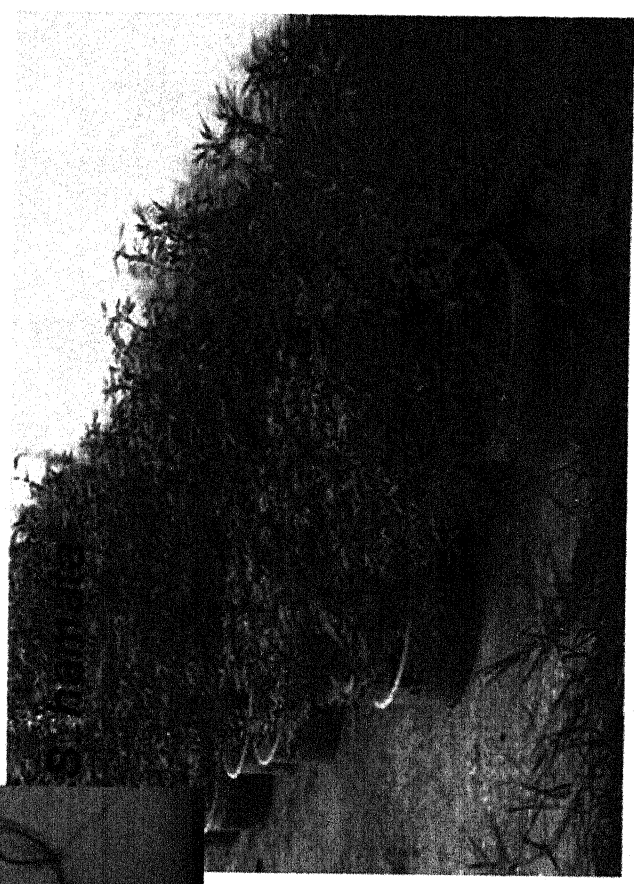
Random primers (10bp) were procured from Operon Inc. USA, and were dissolved in HPLC grade water and stored at -20°C.

## Methodology:

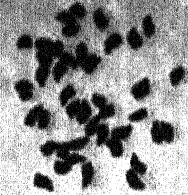
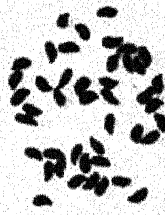
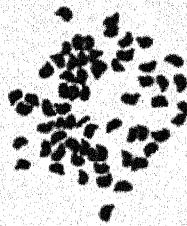
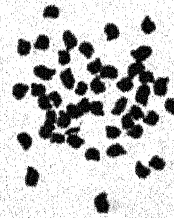
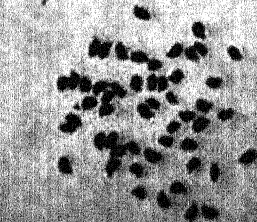
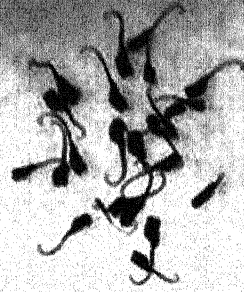
Observations were recorded as per the treatments and three years data were pooled. Standard methodologies with suitable modifications were employed to carry out the experiments.

**Table 1.** List of accessions used and their details

Accessions/Species	Collector, breeder and collection site	Genome
<i>S. seabrana</i>		
IG 369, IG 387, IG 391, IG 370, IG 346, IG 355, IG 325, IG 339, IG 384, IG 352	Accessions received from the collection assembled at ILRI, Ethiopia through NBPGR, New Delhi.	A
EC 408403	CSIRO, Australia	
EC 408404	CSIRO, Australia	A
EC 408405	CSIRO, Australia	A
CPI 110372 (CIAT 10517)	CENARGEN, 6 Km W. Juayeiro, Brazil	A
CPI 105546B	N. Sousa Coasta, 5 Km E. Cactite, Brazil	A
CPI 104710 (CIAT 10026)	N. Schultz-Kraft, 75 Km W. Seabra, Brazil	A
CPI 2523	L.A. Edye, Brazil	A
CPI 2534	L.A. Edye, Brazil	A
CPI 2539	L.A. Edye, Brazil	A
<i>S. scabra</i>		
RRR94-97, Fitzroy, RRR94-96, Q10042, RRR94-86, 36260, RRR94-100, RRR94-93, CPI 40292, Seca.	Brazil	AB
<i>S. viscosa</i>		
CPI 34904, CPI 34941	-	B
<i>S. hamata</i>		
CPI 110123, CPI 110135, CPI 61670	Venezuela	AC
<i>S. humilis</i>		
IG 110		
<i>S. guianensis</i>		
cv. Oxley		G
<i>S. capitata</i>	Colombia	DE



2: Different species of *Stylosanthes*



3: *S. seabrana*

*S. scabra*

*S. viscosa*

*S. hamata*

*S. humilis*

Observations were recorded in following categories:

- 1) Morphological
- 2) Physiological
- 3) Biochemical studies
- 4) Molecular studies

#### **Morphological parameters:**

Fresh weight, dry weight, plant height and number of branches in *stylosanthes* were recorded from three replications separately for control and different levels of salinity.

#### **Physiological parameters:**

**Measurement of osmolality:** Osmolality was measured using Vapor Pressure Osmometer model 5500 (Wescor, USA). The cell sap required for the same was extracted in overnight kept leaves at  $-20^{\circ}\text{C}$  using LP-27 Markhart leaf press model LP-27 (Wescor, USA).

**Chlorophyll and carotene:** Twenty five mg leaves were dipped in 5 ml dimethyl sulphoxide (DMSO) and kept overnight in dark. The optical density (O.D.) was recorded at four wavelengths (663, 654, 510 and 480 nm). Chlorophyll a, b and total carotene were calculated using Arnon's formulas (Arnon, 1949).

**Chl a:**  $(12.7 \times \text{OD}_{663}) - (2.69 \times \text{OD}_{645}) \times 0.2$

**Chl b:**  $(22.9 \times \text{OD}_{645}) - (4.68 \times \text{OD}_{663}) \times 0.2$

**Carotene:**  $(7 \times \text{OD}_{480}) - (1.47 \times \text{OD}_{510})$

**Water potential:** Seedling water potential was measured with uniform discs in three replications from three seedlings of single genotype collected from different levels of salt stress. Using HR 33T due point micro voltmeter, Wescor, USA. Sample chambers (C-52), Wescor, USA were used to put the sample from the middle portion of the leaves of

the size of 2.5 x 2.5mm length and breadth. When the switch of HR33T dew point micro voltmeter was turned to cool position, the temperature of the thermocouple fell below the point temperature and when it was turned to dew point, the temperature converged to the dew point. Water potential was recorded which is linear function of electro magnetic force produced by the temperature difference between the junction at the dew point temperature and the ambient temperature. The recorded value was later divided by proportionality constant ( $-0.75\mu$  moles /bar) to get the value of water potential in bar.

### **Biochemical parameters:**

#### **In vitro study of *Stylosanthes* seedlings:**

The present work was carried out to see the response of different genotypes of *Stylosanthes* to varying levels of salinity under in vitro condition.

**A.1. Prerparation of M. S. media:** Nutrient medium containing inorganic salts, vitamins, growth regulators a carbon source and a gelling agent was used. M S media (Murashige and Skoog, 1962) was used for the germination, and to visualize the effect of salt on seedlings growth. Inorganic salts were mixed in double distilled water supplemented with 0.3% sucrose and solidified by 0.7% agar. The prepared media was separated in five different parts. First as control, left parts were mixed with salt to get 50mM, 100mM, 150mM NaCl and 200mM NaCl concentration in the medium.

The prepared media (20 ml) was poured in each 100 ml conical flask and plugged with non absorbent cotton and wrapped in muslin cloth. The medium was autoclaved for 25 min at 15 Lbs/sq. inch pressure. The autoclaved medium was allowed to cool for overnight. After the solidification at room temperature the media was used for inoculation of seeds of different genotypes.

**A.2. Inoculation of seeds:** Healthy seeds of the respective genotypes were surface sterilized by immersing in 0.1%  $\text{HgCl}_2$  for 1.5-2.0 min followed with 4-6 washings in sterile distilled water. Ten surface sterilized seeds were inoculated in each conical flask in

all the four salt supplemented M S media and control (normal media). These were incubated at  $25 \pm 2$  °C.

**1. Starch gel electrophoresis:** Horizontal starch gel electrophoresis technique discontinuous buffer system was employed with suitable modifications. Among many combinations, 14% starch gel produced best results. In 160 ml of Tris buffer (pH 8.6), 22.4g hydrolysed potato starch was mixed. It was heated with vigorous shaking till it became less viscous and translucent. The cooked starch was immediately poured on to a glass plate in such a way that no air bubbles were trapped. The gel plate was kept to set at room temperature for 5 to 6 hours. Bridge buffer was poured into the buffer chambers so that electrodes were completely dipped. The plates were connected with the buffer chamber with the help of filter paper (Whatman No.1), which was earlier saturated with buffer. In the mean time the migration chamber was placed in a refrigerator to avoid over heating of the gel.

**2. Loading of samples:** The cover from the gel plate was removed with the help of a blade. The sample to be analyzed were soaked on to filter paper wicks (Whatman No.3) of size of 5 x 8 mm and inserted into the slates already made in the middle of the gel for peroxidase and for other isozymes at one side of the gel with the help of the fine cutter. Electrophoresis was carried out at constant current of 24 mA for first 30 minutes followed with 34 mA till the front reaches the other end. The gel plate was taken out and the glass strips were removed from the slide of the glass plates. Each gel was sliced thrice, horizontally, with the help of copper wire to obtain four slices of gel. The first slice was rejected and rests were used for staining. Photographed the gel when bands were properly visualized on gel.

**3. Preparation of polyacryamide gels for electrophoresis:** Thoroughly clean and dry (notch and unnotch) glass plates and spacers, then assemble them properly, spacers should be of same thickness. Held the assembly together into Genei horizontal gel electrophoresis migration chamber and tightly fixed with screw. 2% agar (melted and boiled) is then applied around the edges of the spacers to hold them in place and seal the

chamber between the glassplates. Allowed the agarose to solidify, resulting the sealing of bottom of glass plates. A 40 ml of resolving (separating) gel was prepared by mixing the various constituents and pour between the space of glassplates. Add TEMED and APS in to the buffer at the time of pouring.  $\frac{3}{4}$ <sup>th</sup> space should be filled by resolving buffer and left upper  $\frac{1}{4}$ <sup>th</sup> space for stacking gel buffer. 4-5 drops of butanol at some distance was layered on the resolving buffer. Gel was left for 1 hour min at room temperature for polymerization. After polymerization, over layered butanol was washed off with 3-4 times with distill water, drained completely all the water from the top of the gel, remaining water was soaked with blotting paper. Comb of same thickness as spacers are properly placed at the middle of the notched plate. Stacking gel buffer was prepared by mixing gel components and pour on to the gel casting system from the side of the comb. Let it polymerized for 30 minutes. Remove comb from the gel and wells were washed with distilled water and sealing agarose was removed by displacing spacers from little upper side. Gel plate was now installed in electrophoresis apparatus. Filled the top and bottom reservoir of apparatus with running electrode buffer. Any bubble that trapped at the bottom of the gel between glass plates was removed carefully by tilting the apparatus.

#### 4. Composition of resolving gel buffer (12%):

Gel constituents	Volume
Water	14.0 ml
Tris-buffer (pH 8.9)	10.0 ml
Stock acrylamide solution (30%)	16.0 ml
TEMED	20 $\mu$ l,
APS(10%)	200 $\mu$ l
Total	40 ml

**Composition of resolving gel buffer (10%):**

<b>Gel constituents</b>	<b>Volume</b>
Water	18.0 ml
Tris-buffer (pH8.9)	8.0 ml
Stock acrylamide solution (30%)	13.3 ml
TEMED	20 $\mu$ l
APS (10%)	100 $\mu$ l
Total	40 ml

**Composition of resolving gel buffer (16%):**

<b>Gel constituents</b>	<b>Volume</b>
Water	8.58 ml
Tris-buffer (pH 8.9)	10.0 ml
Stock acrylamide solution (30%)	21.3 ml
TEMED	20 $\mu$ l
APS (10%)	100 $\mu$ l
Total	40 ml

**Composition of stacking gel buffer (4%):**

<b><i>Gel constituents</i></b>	<b>Volume</b>
Water	6.2 ml
Tris-buffer(pH6.7)	2.5 ml
Stock acrylamide solution (30%)	1.3 ml
TEMED	10 $\mu$ l
APS(10%)	50 $\mu$ l
Total	10 ml

### Composition of stacking gel buffer (5%):

<i>Gel constituents</i>	<b>Volume</b>
Water	6.5 ml
Tris-buffer(pH6.7)	1.3 ml
Stock acrylamide solution (30%)	1.7 ml
TEMED	10 $\mu$ l
APS(10%)	50 $\mu$ l
Total	10 ml

### 5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE):

Sodium dodecyl sulphate is an anionic detergent, which binds strongly to, and denatures proteins. The number of SDS molecules bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein –SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the protein. The procedure of gel preparation and casting was same as mentioned in native gel electrophoresis except that in the gel solution SDS was added in both resolving and stacking gel. Similarly in samples, SDS loading dye was added in 1:1 ratio. The mixture was heated in water bath for 2-3 minutes and kept on ice until it is loaded. The gel was run as the native gel. Gel was removed and fixed in 10% TCA for 2-3 hour and then stained as native gel.

### 6. Composition of SDS resolving gel buffer (12%):

<b>Gel constituents</b>	<b>Volume</b>
Water	14.0 ml
Tris-buffer (pH8.9)	10.0 ml
Stock acrylamide solution (30%)	16.0 ml
SDS (10%)	400 $\mu$ l
TEMED	20 $\mu$ l
APS(10%)	100 $\mu$ l
Total	40 ml

### Composition of SDS stacking gel buffer (5%):

Gel constituents	Volume
Water	6.9 ml
Tris-buffer (pH8.9)	1.3 ml
Stock acrylamide solution (30%)	1.7 ml
SDS (10%)	100 $\mu$ l
TEMED	10 $\mu$ l
APS(10%)	25 $\mu$ l
Total	10 ml

**Preparation of enzyme extract for isozyme studies and PAGE:** Fresh young seedlings of control and stress were collected and ground in ice cooled pestle mortar. The tissues were ground (500mg/1ml, w/v) in cooled extraction buffer. The extract was centrifuged at 12000 rpm for 20 minutes. Supernatant was collected and kept at 4°C. The extracted samples were used for native protein, SDS PAGE and isozyme analysis.

**Running of gels:** In each well around 100  $\mu$ g proteins (40-50 $\mu$ l) were loaded after mixing them with 5 $\mu$ l loading dye. Gels were usually electrophoresed at low temperature or at 4°C to resolve isozyme. Ran the gel initially at 5 minutes at 200 volt and after that at 100 volt until the samples have traveled through the stacking gel. After this it was ran again on 200 volt until the bromophenol blue reached the bottom of the gel. After the run was completed, carefully removed the gel between the plates and immersed in 100 ml staining solution and was left for overnight for complete staining. Staining solution was removed and gel was de-stained to remove the dye, which did not bind to proteins. The proteins fractionated on gels were seen as blue colour bands.

### Isozyme studies:

The samples isolated for the study of native and enzyme analyses were electrophoresed using either polyacrylamide buffer (non denaturing) slab gel at a low temperature (4°C)

or starch gel. Each lane was loaded with equal amount of proteins (100 µg) and the volume of the samples loaded were kept in the range of 25-50µl. Gel was run as the native gel but at 4°C. After the run gel was incubated in a solution containing all the necessary components for enzyme reaction for different isozyme analyses (given below). Different isoforms of the enzyme were noticed in form of coloured bands. The study of isozymes was carried out following the vertical polyacrylamide gel (12%) electrophoresis method (Laemmli, 1970) with discontinuous buffer system. Seven enzymes namely LAP, FBA, PPO, GOT, CAT, PGM and IDH were resolved in 14% starch gel.

### **Gel electrophoresis:**

Isozyme patterns of LAP, FBA were performed using enzyme source obtained with crushing buffer-A, tray buffer (0.028 M LiOH, 0.192M borate pH 7.9), gel buffer (0.002 M LiOH, 0.019M borate, 0.04 M Tris, 0.04 M citrate pH 7.9) and crushing buffer B using tray buffer (0.036M Tris, 0.01M citrate pH 6.8), gel buffer (diluted tray buffer in 1:3 with water) for the isozyme patterns of PPO, GOT, CAT, PGM, IDH. Isozyme patterns of these seven enzyme systems were carried out using 14 % starch gels. Crushing buffer C was used for EST, SOD, MDH, ACP, POD, AMY and GDH enzymes using 12 % polyacrylamide gels. About 150 µg of protein sample mixed with 5 µl of bromophenol blue (tracking dye) was loaded onto the well from cathodal end. The gels were run at 100 V till dye crossed the stacking gel and then at 200 V till the dye was ½-1 cm away from bottom. In starch gel, samples were loaded in the form of soaked filter paper (5 x 10 mm) in enzyme extract and placed on the slot made onto the starch gel and electrophoresis was performed at 4°C at 50 mA for 4 hrs. Starch gels were sliced and incubated in staining media at 37°C in the dark until the bands appeared. The gels were stained as described by Chao and Gale (1990) for peroxidase and as described by Wendel and Weeden (1989).

**(a) Esterase:** Gel was immersed in 100 ml 0.5 M sodium phosphate buffer and incubated at room temperature for 30 min. In an Eppendorf tube 50 mg of alpha naphthyl acetate was dissolved in 1 ml of 60% acetone and in the same tube 50 mg fast blue RR salt was

dissolved and poured in already incubated gel. The gel was now incubated at 37°C for 20-30 minutes in dark. The reaction was stopped by adding the mixture of methanol, acetic acid, water and ethyl alcohol in the ratio of 10:2:10:1. The gel was photographed using Olympus OM 2000 camera.

**(b) Peroxidase:** For peroxidase the gel was incubated in a 100ml staining solution containing 2.50gm catechol, 1.96gm Tris, 0.150gm boric acid 0.190gm EDTA and 1.50gm CaCl<sub>2</sub>, after 30 minutes transfer the gel in 100ml of 0.01M H<sub>2</sub>O<sub>2</sub> solution. Left the gel in staining solution until black bands were appeared on upper side of the gel. Remove stain after proper bands visualization.

**(c) Polyphenol oxidase:** When the bromophenol blue dye touched the bottom of the gel it was removed and equilibrated in 100 ml 0.1M potassium phosphate buffer (pH 7.0) containing 0.1% p-phenylenediamine for 30 minutes at room temperature. When the incubation was over, catechol was added to final concentration of 0.1% in the same buffer (Jayaraman *et al.*, 1987). Dark brown bands appeared were photographed using OM 2000 camera.

**(d) Acid phosphatase:** After removing the gel from gel assembly it was washed thoroughly 3-4 times in 0.1 M acetate buffer (pH 5.0) so that pH of gel came down to 5.0. Incubated the gel at 37°C for 2-4 hours in 100 ml of 0.1 M acetate buffer containing 50 mg 1- Naphthyl phosphate, 50 mg Fast blue RR (dissolved in 60% acetone), 1.0 gm Sodium chloride and 100 mg Magnesium chloride. Reddish-brown bands appeared which was later fixed in 50% ethanol (De and Roy, 1984).

**(e) Amylase:** Amylase bands are revealed as translucent areas or bands in the opaque starch matrix on gels stained for other enzymes particularly those involving tetrazolium salts like NBT or MTT, PMS and Fast Black K salt. After the gel run, left it overnight at room temperature to enhance band development. Regions of amylase activity are translucent or "clear", and should not be confused with achromatic regions resulting from the activity of superoxide dismutase. Amylases are usually studied on polyacrylamide

gels using an iodine-potassium iodide assay (Vallejos, 1983). As discussed by Vallejos,  $\alpha$ - and  $\beta$ -amylases may be distinguished by differential sensitivity to heat, pH and heavy metal ions.

**(f) Catalase:** After running of starch gel to the end, pour 0.01% 100 ml hydrogen peroxide solution and leave the gel for 10-15 minutes. Meanwhile mix 1gm ferric chloride and 1 gm potassium ferricyanide in 100 ml distill water. Pour off peroxide solution and add stain solution in to the gel. Swirl gently until bands are developed. Catalase activity was revealed as achromatic zones on a green background. After band formation rinse the gel 3-4 times and store it in distill water. It is best to photograph and scored it shortly after staining.

**(g) Superoxide dismutase:** When the run was over gel was removed from the gel assembly and incubated in dark for 30 minutes in 100 ml of 0.05 M Tris buffer (pH 8.0) containing 2 mg riboflavin, 1 mg EDTA and 10 mg NBT. After the incubation gel was shifted to bright and intense light for 30 minutes and then gel was washed with distilled water. The bands appeared in form of negative bands against the blue background.

**(h) Phosphoglucose isomerase:** The gel was incubated in 100ml Tris-HCl buffer pH-8.0 containing 20mg NAD, 40mg fructose-6-phosphate ( $\text{Na}_2$  salt), 40mg glucose-6-phosphate dehydrogenase (NAD), 20mg MTT and 4mg PMS. Pour the staining solution over gel. The gel was further incubated at room temperature for 3 hrs until blue intense bands were appeared in the gel.

**(i) Alcohol dehydrogenase:** After the run, gel was incubated in 100 ml of 0.05 M Tris buffer (pH 8.0) containing 0.5 ml absolute alcohol at room temperature for 30 minutes. In a 1.5 ml Eppendorf tube, 10 mg NAD, 10 mg NBT and 2 mg PMS was dissolved in 1 ml Tris buffer and added in the gel. The gel was further incubated at 37°C for 3-4 hrs and bands appeared were photographed using OM 2000 SLR camera.

**(j) Acid phosphatase:** Gel 3-4 times washed in 0.1M acetate buffer (pH-5) by changing the buffer every 15 minutes (to lower the pH of the gel to 5.0). Incubate the gel at 37°C for 2-3h in the 100 ml buffer containing 0.10 gm 1-Naphthyl phosphate, fast blue RR 0.10 gm, 1.0 gm sodium chloride 1.0 ml magnesium chloride (10%). Incubate until reddish brown bands were visible in the gel. Fix the gel using 50% ethanol.

**(k) Leucine aminopeptidase:** After the gel was run placed it in 100ml, 200mM Tris-200mM maleate pH 3.7 buffer over gel. Dissolved L-leucyl- $\beta$ -naphthylamide.HCL (substrate) and fast black K salt in the dimethylformamide and added over gel.

**(l) Malate dehydrogenase:** Gel was incubated in 50mM Tris-HCl, pH 8.5 buffer containing NAD 10mg, neutralized 150mg malic acid, 10mg NBT and 2mg PMS. Ingredients were combined and pour over gel and incubated in dark until blue bands appeared.

**(m) Isocitrate dehydrogenase:** In 50 ml of 50mM of Tris-Cl pH-8.0, 600mg of agar was added. Boil this solution and wait to cool down the solution to 60°C. In another 50ml, 50mM Tris-HCl, pH-8.0 50mg MgCl<sub>2</sub>, 10mg NAD(P), 100mg isocitric acid, 10mg NBT and 2 mg PMS was added. Gently mix the ingredients in to the buffer. Mixed solution A in solution B and poured this solution over gel. Once agar has solidified gel was incubated until blue bands appeared.

**(n) Glucose oxaloacetate transaminase:** Substrate solution was prepared for the staining of GOT. In 80 ml distilled water, 29.2 mg  $\alpha$ -ketoglutaric acid, 0.107 g L-Aspartic acid, 0.4 gm PVP-40, 40mg EDTA, Na<sub>2</sub> salt and 1.36 gm sodium phosphate, dibasic was dissolved. Mix well all ingredients and poured over gel. After ten minutes 20 mg Fast blue BB salt was mixed in 20 ml substrate solution and poured over gel. Incubated the gel at room temperature in the dark, until blue bands appeared.

**Construction of zymogram:** The bands for different isozymes were drawn on graph sheet in 1:1 ratio. The point of origin and front was also marked in order to calculate

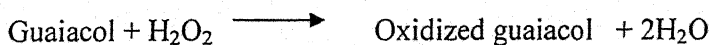
relative mobility of the bands. Relative mobility of the band was calculated by dividing the distance traveled by the respective bands from the origin of resolving gel by the distance traveled by dye from the origin of resolving gel. Microsoft excel program was used to construct the zymogram.

**Nomenclature of bands:** The bands were scored from the plate of origin where sampled were loaded to the end of front/dye movement. The slowest band was considered as bands 1 and subsequently bands were numbered.

#### **Assay of enzymes activities:**

**Preparation of enzyme extract:** Leaves were collected in icebox and were ground using pre cooled pestle and mortar until no fibrous residue was seen. The grinding medium was 0.1M potassium phosphate buffer pH-7.0 used in 1:2(w/v) with seedlings. The homogenate was centrifuged at 4°C for 20 min. at 10,000 rpm. The supernatant was referred as enzyme extract, and used for the assay of enzyme activity. Used the supernatant, as enzyme source within 2-4 hrs for superoxide dismutase, peroxidase and catalase. Rest of the extract was stored in ice till the assayed was carried out. An aliquot of the extract was used for protein determination (Lowry *et al.*, 1951) and used to determine the specific activity of the enzymes.

**Determination of peroxidase activity:** It was measured by the method of Chance and Machly (1955) where guaiacol was used as the substrate



The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product was measured as the POD activity. In a 5 ml cuvette, 3 ml 0.1 M potassium phosphate buffer containing, 0.1 ml 20 mM guaiacol solution, 0.03 ml 12.3 mM of hydrogen peroxide and 0.05 ml of enzyme extract was taken. Mixed well and

placed the cuvette in the UV-VIS spectrophotometer against the reference where same solution except enzyme extract was present. Increase in absorbance with time was recorded at 436 nm. This was done till the straight line appeared. Activity was determined as change in 0.01 OD per minute (one unit). It was divided by fresh weight content in mg present in aliquot to get the peroxidase activity (unit/mg fr. wt.).

**Determination of superoxide dismutase activity:** The SOD enzyme activity was measured as its ability to inhibit photochemical reduction of NBT and was carried out using the method of Giannopolitis and Ries (1977) with suitable modifications. Reaction was carried out in 3 ml of solution containing 0.05M Na<sub>2</sub>CO<sub>3</sub>, 0.1 mM EDTA, 63μM NBT, 13μM methionine, 20μl enzyme extract and 1.3μM riboflavin. The riboflavin was added at last. The test tubes were placed under two 40W fluorescent lamps at a distance of 30 cm at 25°C. After 15 minutes the light was switched off and the absorbance at 560 nm was recorded. The non-irradiated sample without enzyme extract served as blank. Absorbance obtained with sample containing enzyme extract and kept in light was deducted from the absorbance obtained with the sample without extract and kept in light. The reaction mixture lacking enzyme extract developed maximum colour due to maximum reduction of NBT. The reduction of NBT found inversely proportional to the enzyme activity.

#### **Determination of catalase enzyme activity:**

Catalase has a double function as it catalyzes the following reactions:

1. Decomposition of hydrogen peroxide to give water and oxygen



2. Oxidation of H donors for example methanol, formic acid, phenol with the composition of one mole of peroxide



Catalase activity was carried out in freshly ground tissues using 3 ml of phosphate buffer containing 5  $\mu$ l of 15% hydrogen peroxide. In the same tube 10  $\mu$ l ml of enzyme extract was added and mixed well. Decrease in optical density was recorded against the reference where same solution except enzyme extract was present at 240 nm. Activity was determined and presented as change in 0.01 OD per minute (one unit). It was divided by fresh weight content in mg present in aliquot to get the catalase activity (unit/mg fresh weight).

**Glutathione reductase:** In a 3 ml reaction mixture, take 2.86 ml 0.025mM pH 8.0 sodium phosphate buffer and 30 $\mu$ l G-S-S-G from 50mM stock was mixed. In the same mixture 14 $\mu$ l of NADPH.Na<sub>4</sub> from 50mm stock was added. Finally 100 $\mu$ l of enzyme extract was added in the reaction mixture. O.D. at 340 nm was recorded for 5 minutes at 1 min intervals. It was calculated in unit/mg fr. wt. /min.

**Glycolate oxidase:** In 1 ml reaction mixture, 850 $\mu$ l of NaPO<sub>4</sub> buffer from 0.5M buffer stock, 15.2 $\mu$ l of catalase from 1.317unit/ $\mu$ l stock and 30 $\mu$ l enzyme extract was mixed in a centrifuge tube. It was incubated at 30°C for 3 minutes. In the same tube 100 $\mu$ l sodium glycolate from 50mM stock was added. Incubated at 30°C for 10 minutes and then terminated the reaction by adding 100 $\mu$ l of 2N HCl. Centrifuged it 10,000 rpm for 5 minutes. One ml reaction mixture was taken and added 100 $\mu$ l 1.82M NaOH. One ml neutralized sample was taken and added 200 $\mu$ l 0.33% phenyl hydrazine (or 100 $\mu$ l from 3.3% stock). Incubated at 30°C for 10 min. In ice cold condition 200 $\mu$ l of both concentrated HCl and 1.65% potassium ferricyanide was added in the reaction mixture. Mixed well and left for 15 minutes to develop the color and then O.D. was recorded at 550 nm. It was expressed in unit/mg fr. wt. /min

**Ascorbate peroxidase:** In a 3 ml reaction mixture, 2 ml 0.1M (pH-7.0) potassium phosphate buffer, 5 $\mu$ l hydrogen peroxide and 25 $\mu$ l of ascorbate from 25mM stock was mixed in the same tube. 50 $\mu$ l of enzyme extract was added, mixed well and O.D. was recorded at 290 nm for 5 minutes at 1 min of interval. It was calculated in unit/mg fr. wt. /min.

### **Estimation of malondialdehyde:**

It was estimated in leaf tissues and seedlings by taking two hundred fifty milligram samples and ground in 5 ml distilled water. In the same solution 5 ml TBA-TCA reagent (0.5% thiobarbituric acid and 20% TCA dissolved in 100 ml of distilled water) was added. The slurry was kept in water bath at 95°C 30 min. Cooled the solution by placing in ice bath and centrifuged at 10,000 rpm for 10 min. Collected the supernatant carefully and recorded the OD at 600 nm and 535 nm. The difference in optical density gave the actual intensity of colour developed by malondialdehyde (MDA). It was calculated in nanogm/gm fr. wt.

### **Estimation of proline:**

Proline, a basic amino acid reported to play important role in stress conditions. It was estimated following the method of Bates *et al.*, (1973). During selective extraction with aqueous sulphosalicylic acid, proteins were precipitated as a complex. Other interfering materials were also removed by absorption to the protein-sulphosalicylic acid complex. To estimate proline from leaves and seedlings 0.5g of plant material was homogenized in 10 ml of 3% aqueous sulphosalicylic acid. Filtered the homogenates through Whatman No. 2 filter paper. Took 2 ml of filtrate in a test tube, added 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin (1.25g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid, with agitation until it is completely dissolved, stored at 4°C and used within 24h). Heated the mixture at 100 °C for 1 hour. Terminated the reaction by placing the tube in ice bath. Added 4 ml.toluene to the reaction mixture and stirred well for 20-30 sec. Using the separating funnel the solution was extracted and toluene containing proline was collected. Measured the developed red colour intensity at 520nm. Proline content of plant material was recorded against toluene used as blank.

### **Estimation of total soluble protein:**

Protein was estimated from seedling extract prepared for isozyme analysis, following the Lowry method (Lowry *et al.*, 1951). In one ml of water 10  $\mu$ l enzyme extract was taken and in the same test tube 5 ml of reagent C was added to each tube. After 10 min. of incubation 0.5 ml of Folin–Ciocalteu reagent was added and mixed well. All tubes were incubated at room temperature in dark for 30 min. Blue color developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin–Ciocalteu reagent by the amino acids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartrate was measured.. In blank instead of enzyme extract water was taken. O.D. was recorded at 660 nm of the color developed from reaction.

### **Isolation of plant DNA:**

To carry out the polymerase chain reaction (PCR) there is a need to have enough amount of good quality of genomic DNA. Genomic DNA was isolated from whole seedlings using buffer 'S' (100 mM Tris-HCL pH 8.0, 50 mM EDTA, 100 mM NaCl and 2 % SDS) in 1:2 ratios. Fine powder of leaf samples was made using liquid nitrogen. In case of mini prep, 4-5 seedlings were directly ground in 1.5ml eppendorf tubes, in the presence of 500 $\mu$ l buffer 'S' or 200mg fine powder was mixed with 1ml buffer 'S' and it was incubated at 65°C for one hour with occasional swirling. In each tube 400 $\mu$ l of 5M sodium acetate was added. Mixed well for 2-4 times and kept on ice for 30 min. It was centrifuged at 8000 rpm for 10 min and upper clear liquid phase was collected in fresh tubes. Nucleic acid was precipitated with 0.6 volume of isopropanol. It was centrifuged at 8000 rpm for 10 min and was rinsed in 300 $\mu$ l of 70 % ethanol and finally dissolved in 500 $\mu$ l of TE buffer. It was further treated with RNase (6 $\mu$ l/ml) and after 30 min of incubation at 37 °C, DNA was extracted twice with phenol-chloroform (400  $\mu$ l each) and finally only with chloroform. DNA was precipitated in 100 % ethanol and pelleted at 10000 rpm for 10 min. The supernatant was discarded and DNA was washed with 70 % ethanol and centrifuged at 10000 rpm for 5 min. The DNA pellet was air dried and again

dissolved in 1 ml TE. The quantity and quality was checked on 0.8 % agarose gel in 0.5 %TBE buffer and finally diluted with TE to a concentration of 5ng/μl for STS and RAPD analysis.

#### **Agarose gel electrophoresis of genomic DNA:**

The DNA was electrophoresed on 0.8% agarose gel using 0.5X TBE buffer. After a brief run the gel was stained in ethidium bromide. The brief run was enough to reveal the quality and quantity of DNA. The gel was run at 70 volt using 0.5X TBE buffer. When run was over the gel was photographed using Polaroid and SLR camera.

#### **Polymerase chain reaction and RAPD analysis:**

Polymerase chain reaction was carried out in final volume of 20 μl of reaction mixture containing 67 mM Tris-HCl (pH 8.0), 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.45 % v/v BSA, 3.5 mM MgCl<sub>2</sub>, 150 μM of each dATP, dTTP, dCTP, dGTP, 7.5 pmoles (15ng) primer, 0.5 unit Taq polymerase and 25 ng genomic template DNA. PCR product was visualized with ethidium bromide after electrophoresis on 1.6 % agarose gel.

#### **Composition of reaction mixture for single RAPD reaction (20μl)**

10X PCR buffer <sup>(SM)</sup>	-	2.0μl
MgCl <sub>2</sub> (25mM)	-	2.4μl
dNTPs mix (10mM each)	-	1.4μl
Primer (10mM)	-	1.5μl
Taq DNA Pol(3U/μl)	-	0.2μl (0.5unit)
H <sub>2</sub> O	-	7.5μl
Genomic DNA	-	5.0 μl (Diluted template DNA to 5ng/μl to get 25ng)

Mixed all the components (except genomic DNA) and 15 $\mu$ l of the mixture was added in each single reaction. Genomic DNA should be taken previously in PCR tubes. The reaction mixture was overlaid with one drop of mineral oil in order to avoid evaporation. The amplifications were carried out in MJ Research PTC-200 peltier thermal cycler, programmed for 40 cycles of 94°C for one minute (denaturation), 37°C for one min (annealing) and 72°C for two min (amplification). After completion of 40 cycles, in 41<sup>st</sup> cycle, the reaction was kept on 72°C for 10 min as 41<sup>st</sup> cycle and then hold at 4°C until the tubes were removed from the PCR.

**Agarose gel electrophoresis for RAPD-PCR product:** PCR products were separated on a 1.6% agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2 $\mu$ l 10X DNA loading buffer and were loaded on the gel. Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was run at 70 volts for four hours. The amplified product were visualized under UV trans-illuminator and were photographed were taken using Polaroid and SLR camera.

#### **STS- PCR Analysis:**

For STS-PCR, 17 STS primer pairs used in the present investigation were primarily derived from the *Pst*I clones obtained from *S. scabra* cv Fitzroy and *S. hamata* cv Verano (Vander Stappen *et al.*, 1999) as well as different coding and non-coding regions of gene sequences (Curtis *et al.*, 1995; Liu *et al.*, 1996; Manners *et al.*, 1995; Reddy *et al.*, 1996; Smith *et al.*, 1995). The OLIGO (version 3.0) computer program was used to select the optimal oligonucleotide for STS primer pairs as described by Liu *et al.* (1996). An oligonucleotide was selected only when its T<sub>m</sub> was greater than 50°C and when its 3' terminus was not complementary to itself or to the other primer with which they form a pair. Primers were synthesized by Sigma Genosys Company according to the sequences by Liu and Musial (1995) and Vander Stappen *et al.* (1999). The polymerase chain reaction (PCR) amplification for STS loci followed the procedure described by Liu *et al.* (1996) with modification in genomic DNA concentration and inclusion of primer extension step for 5 min as a last step of amplification.

The total reaction volume was 25  $\mu$ l, which contained 78.2 mM Tris-HCL (pH 8.8), 19.4 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.53 % (v/v) Triton X-100, 233  $\mu$ g/ml BSA, 5.8 mM  $\text{MgCl}_2$ , 130 mM dNTPs, 0.3  $\mu$ M of primers, 1.2 unit of Taq DNA polymerase (Bangalore Genei, India) and 25 ng of genomic DNA templates. The reactions were overlaid with mineral oil. The PCR protocol was performed in an MJ Research PTC-200.

#### **Composition of reaction mixture for single STS-PCR (25 $\mu$ l)**

10X PCR buffer <sup>(SM)</sup>	-	3.3 $\mu$ l
$\text{MgCl}_2$ (25mM)	-	6.6 $\mu$ l
dNTPs mix (10mM each)	-	2.9 $\mu$ l
Primer (10 $\mu$ M)	-	2.0 $\mu$ l (1.0 $\mu$ l Reverse and Forward primer each)
Taq DNA Pol (3U/ $\mu$ l)	-	0.5 $\mu$ l (1.0 unit)
$\text{H}_2\text{O}$	-	13.4 $\mu$ l
Genomic DNA	-	5.0 $\mu$ l (Diluted template DNA to 5ng/ $\mu$ l to get 25ng)
Final volume	-	25 $\mu$ l

Mixed all the components (except genomic DNA) and 20 $\mu$ l of the mixture was added in each single reaction.

#### **Following program was used in PCR amplification for STS loci.**

94°C	- 60 second
55°C	- 60 second
72°C	- 90 second

Repeat above steps for 32 cycles

One cycle at 72°C for 5 min and finally 4°C for ever. Amplified products were stored at 4°C.

**Agarose gel electrophoresis for STS-PCR product:** STS-PCR products were separated on a 1.6% agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2ul 10X DNA loading dye and were loaded on the gel. Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was run at 70 volts for four hours. The amplified product were visualized under UV trans-illuminator and were photographed were taken using gel documentation system.

**Analysis of amplification profiles:** In all the cases the consistency of bands was checked by running the reaction twice, and only the reproducible bands were scored in all samples. For RAPD and STS markers input binary data matrix of *Stylosanthes* accessions were developed by entering the data by assigning 1 to presence or 0 to absence of bands. The NTSYS program version 2.0 was used to produce the similarity matrix (Simqual function) for both isozyme and STS data. Dice similarity coefficient was used to estimate the genetic similarity. The resulting data were further processed for SAHN cluster analysis using the un-weighted pair group average method (UPGMA) and tree display was followed for generation of dendrogram (NTSYS tree phenogram). Polymorphic information content (PIC) value was calculated of individual primer to evaluate discriminatory power of RAPD. The PIC value was calculated applying the formula of Roldan-Ruiz *et al.* (2000):  $PIC_i = 2f_i(1-f_i)$ , where  $f_i$  is the frequency of the amplified allele (band present) and  $(1-f_i)$  is the frequency of the null allele (band absent) of marker  $i$ . Marker index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell *et al.*, 1996). Level of polymorphism, frequencies obtained with STS and RAPD were calculated following the standard statistical methods (Gomez and Gomez, 1984). The reliability of the generated dendrogram was also tested by bootstrap analysis using WinBoot software (Yap and Nelson, 1996) with 1,000 iterations.

## Isolation of plant RNA

To isolate total RNA from young and fresh seedlings, immediately freeze seedlings in liquid nitrogen or store at  $-20^{\circ}\text{C}$  in RNA stabilizing agent (1mM sodium citrate pH-6.4) was used. Isolation of total RNA from the fresh seedlings was carried out using QIAGEN RNeasy plant mini kit. Glasswares were treated before use to ensure it was RNase-free. Glassware used for RNA was cleaned with a detergent, thoroughly rinsed, and oven baked at  $240^{\circ}\text{C}$  for four hours. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware was treated with DEPC (Diethyl pyrocarbonate). All glassware were immersed in DEPC (0.1% in water), allowed to stand overnight (12 hours) at  $37^{\circ}\text{C}$ , and then autoclaved or heat to  $100^{\circ}\text{C}$  for 15 minutes to eliminate residual DEPC. The procedure of RNA isolation was as followed:

1. Ground seedling sample in liquid nitrogen in a DEPC treated and autoclaved, pre-chilled pestle and mortar
2. Took 100mg of fine powdered seedling tissues in a 1.5ml eppendorf tube and added 450 $\mu\text{l}$  of RLT buffer along with 4.5 $\mu\text{l}$   $\beta$ -Mercaptoethanol (ME).
3. Mixed the sample vigorously by vortexing. Transferred the lysate into a QIA shredder spin column (lilac colored column) placed in a 2ml collection tube.
4. Centrifuged for 2 min at 12000 rpm at room temperature  $20-25^{\circ}\text{C}$ .
5. Carefully transferred the flow through fraction of supernatant in a new eppendorf tube without disturbing the cell debris pelleted in the collection tube.
6. Added 0.5 volume of ethanol (96-100%) to the clear lysate and mixed well by pipetting up and down.
7. Placed immediately the sample into RNeasy mini column (pink colored column) placed in 2ml collection tube.
8. Centrifuged at 12000 rpm for 15sec at RT.
9. Discarded the flow through and retained the collection tube.
10. Took 350 $\mu\text{l}$  of buffer RW1 and placed into the RNeasy column.
11. Centrifuged at 12000 rpm for 15 sec at RT.

12. Added 80µl of DNase 1 and incubated the mixture (containing 10 µl stock DNase1 solution+ 70µl buffer RDD) onto the RNeasy silica gel membrane and incubated at 20-30°C for 15-20 min.
  13. Took 350µl of buffer RW1 and placed into the RNeasy column.
  14. Centrifuged at 12000 rpm for 15 sec at RT.
- Note: if using steps no 10-14, did not use steps no 15-16, directly stepped to step no 17.
15. Took 700µl of buffer RW1 placed into the RNeasy column.
  16. Centrifuged at 12000 rpm for 15 sec at RT, discarded the flow through and collection tube.
  17. Transferred the RNeasy column to a new 2ml collection tube and added 500µl of buffer RPE (ensure that ethanol had been added to the buffer).
  18. Centrifuged at 12000rpm for 15 sec at RT.
  19. Dried the membrane of the column by centrifuging again at 12000 rpm for 1 min.
  20. Transferred the RNeasy mini column to a new 1.5ml eppendorf tube
  21. Applied 30µl of RNase free water containing RNase-inhibitor @ 0.5units/µl into the column and centrifuged at 12000rpm for 1 min.
  22. Again loaded the flow through into the same column and centrifuged at 12000rpm for 1 min (for better elution).
  23. In seedlings purification of RNA sample by phenol-chloroform extraction was not needed.
  24. If needed quantified the RNA spectrophotometrically by taking OD at 260/280 nm.

#### **Agarose gel electrophoresis of genomic RNA:**

The RNA was electrophoresed on 1.0% agarose gel using 50X TAE buffer. 2µl of sample was mixed with 2µl 6X RNA loading dye solution and loaded on gel. After a brief run the gel was stained in ethidium bromide or it was added previously at the time of gel making. The brief run was enough to reveal the quality and quantity of RNA. The gel was run at 50 volt using 50X TBE buffer. When run was over the gel was photographed using Polaroid and SLR camera. 5µl of RNA ladder, low range, 0.25mg RNA/ml, 0.01mg was used as a marker with RT-PCR products.

## RT-PCR analysis

One step RT-PCR buffer was designed to enable both efficient reverse transcription and specific amplification. The unique buffer composition allows reverse transcription to be performed at high temperatures (50°C). This high reaction temperature improves the efficiency of the reverse-transcriptase reaction by disrupting secondary structures and is particularly important for one step RT-PCR performed with limiting template RNA amounts. Q solution provided with RT-PCR kit was an additive that facilitates amplification of difficult templates by modifying the melting behavior of nucleic acids.

**Buffers and reagents:** One Step RT-PCR Enzyme Mix contained 20mM Tris-Cl, 100mM KCl, 1mM dithiothreitol (DTT), 0.1mM EDTA, 0.5%(v/v) Nonidet P-40, 0.5%(v/v) Tween 20, 50% glycerol (v/v), stabilizer, pH 9.0 (20°C), 5X RT-PCR buffer contained Tris-Cl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 12.5mM MgCl<sub>2</sub>, dithiothreitol (DTT) pH8.7 (20°C), 5X Q solution, dNTP mix, 10mM each of dATP, dCTP, dGTP, dTTP, RNase free water.

### Composition of reaction mixture for single RT-PCR reaction (25µl)

RT-PCR buffer	5.0µl
Q solution	5.0µl
RT-PCR Enzyme Mix	1.0µl
dNTP Mix	1.0µl
RNA	2.0µl
Primer	2.0µl
RNase free water	9.0µl
<hr/>	
Total volume	25µl

Mixture of appropriate quantity of RNA, water and primer (in 0.2 ml PCR tube) was placed on 50°C. In the same tube reaction mixture containing buffer, Q solution, enzyme

mixture and dNTP was added.. The reaction mixtures were kept for reaction in PTC 100 thermocycler (MJ Research, USA) with following reaction cycle:

1. 1st step 50°C for 30 min
2. 2<sup>nd</sup> step 95°C for 15 min
3. 94°C for 45 seconds
4. 48°C for 1 min
5. 72°C for 2 min
6. Repeated 3-5 for additional 29 times
7. 72°C for 10 min
8. 10°C forever

The amplified products were ran on 1.6% agarose gel in 0.5X TBE buffer and visualized under gel documentation system.

#### **Composition of M S Media used in present study:**

Components	Quantity
<b>Macro salts</b>	
NH <sub>4</sub> NO <sub>3</sub>	1.65 gm
KNO <sub>3</sub>	1.90 gm
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.44 gm
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.37 gm
KH <sub>2</sub> PO <sub>4</sub>	0.17 gm
<b>Micro salts</b>	
FeSO <sub>4</sub> .7H <sub>2</sub> O	27.80mg
Na <sub>2</sub> EDTA.2H <sub>2</sub> O	33.60 mg
KI	0.83mg
H <sub>3</sub> BO <sub>4</sub>	6.20 mg
MnSO <sub>4</sub> .4H <sub>2</sub> O	22.30 mg

ZnSO <sub>4</sub> .7H <sub>2</sub> O	8.60 mg
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.25 mg
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025 mg
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025 mg

### Organic Supplements

Myoinositol	100.0 mg
Nicotinic Acid	0.05 mg
Pyridoxine-HCl	0.05 mg
Thiamine-HCl	0.05 mg
Glycine	2.0 mg
Sucrose	20.0 mg

Dissolved the salts and organics in 800 ml of distilled H<sub>2</sub>O. Adjusted pH to 5.7 by adding 1 M NaOH. Added additional distilled water to adjust the final volume to 1 litre. Approximately 10-15 ml of M. S. media was taken in culture tubes and with the help of cotton plug mouth of these tubes was closed. Autoclaved the media for 20 minutes at 15 psi.

### Solutions and buffers for plant DNA isolation:

1) 0.5M EDTA pH 8.0 : Dissolved 37.22g EDTA disodium salt and 4.0g Sodium hydroxide in 150 ml H<sub>2</sub>O and adjusted the pH by NaOH solution, final volume was made up to 200 ml.

2) 1M Tris-Cl pH (8.0) : Dissolved 24.23g Tris-base in 150 ml distilled water Adjust to the pH 8.0 by HCL and made up final volume to 200 ml with water.

3) 5M NaCl : Dissolved 58.44g NaCl in distilled water and made up final volume to 200 ml with water.

4) CTAB (10%) W/V : Dissolved 200g CTAB in 180 ml distilled water. CTAB was dissolved by warming the solution. The final volume was made up to 200 ml.

5) 10mM TE Buffer (pH 8.0) : 2ml Tris-Cl from 1M Tris-Cl stock and 0.04 ml EDTA from 0.5M EDTA stock was taken for buffer. The final volume was made up to 200 ml.

6) 5X Tris-Borate EDTA Buffer or TBE Buffer (pH 8.0) : Dissolved 27g Tris-base, 13.75g boric acid, 10ml (0.5M) EDTA in 400ml distilled water and made to final volume to 500 ml with water.

**7) Buffer 'S' for seedling DNA isolation:**

Final concentration

100 mM Tris-HCL pH 8.0	20ml (1M Tris-Cl pH-8.0)
50 mM EDTA	20ml (0.5M EDTA)
100 mM NaCl	4ml (5M NaCl)
2 % SDS	20ml (20%)

**8) CTAB total DNA Extraction Buffer :**

Final concentration

Stock concentration

20mM EDTA	8ml (0.5M EDTA)
100mM Tris-Cl	20ml (1M Tris-Cl pH-8.0)
1.4M NaCl	56ml (5M NaCl)
2% W/V CTAB	40ml (10% CTAB)
2-Mercaptoethanol	added freshly

Mixed and made up final volume to 200 ml with water.

**9) Loading dye (Bromophenol blue):**

Final concentration

Stock concentration

0.1M EDTA	10ml (0.5M)
40% Sucrose	20g
25% Bromophenol blue	125mg

Final volume was made up to 50 ml with distilled water.

10) 10X Taq buffer:

<u>Stock concentration</u>	<u>Vol./Amt. taken</u>	<u>Final concentration</u>
1M Tris-Cl (pH 6.8)	6.7 ml	0.67M
1M Ammonium sulphate	1.66 ml	0.166M
100% Triton X-100	0.45ml	4.5% (V/V)
BSA	20mg	0.2%

Final volume was made to 10 ml with HPLC grade sterile water.

**Buffer/ solution for PAGE:**

1) Acrylamide stock 30%:

Acrylamide	29.2g
Bis -acrylamide	0.8 g

Dissolved in 80 ml distilled water and made up the final volume to 100 ml water. Filtered the solution and stored at 4°C.

2) Resolving gel buffer pH 8.9: Dissolved 18.15 gm Tris-base in 60 ml distilled water. Adjusted the pH 8.9 by adding HCl and made the final volume 100 ml with water.

3) Stacking gel buffer: (0.6M Tris-base pH 6.7) Dissolved 6.1g Tris-base in 60 ml distilled water and adjusted pH6.7 by adding HCl and made up final volume 100 ml.

4)10% ammonium persulphate solution (APS): Dissolved 100 mg APS in 1ml distilled water.

5) Running electrode buffer pH 8.3:

Tris Base	0.6g
Glycine	2.8g
Water	1 liter

6) Staining solution:

Coomassie brilliant blue-R-250	100 mg
Methanol	40 ml
Acetic acid	10 ml

Made up final volume to 100 ml with distilled water.

7) De-staining solution :

Methanol	40 ml
Acetic Acid	10 ml
Distilled Water	50 ml

**Extraction buffers for isozyme and PAGE analyses:**

**1) Crushing buffer 1**

0.05M Tris buffer	0.605g
Sucrose (5%w/v)	5g
EDTA	168 mg
2-ME	100μl

Dissolved in 60 ml distilled water and adjusted the pH 7.5 by adding 1 N HCl and made up the final volume to 100 ml.

**2) Crushing buffer 2**

Phosphate buffer pH-7.5	0.01M
PVP-40	12%
BSA	0.3%
EDTA	0.03%
2-ME	0.01M

**3) Crushing buffer 3**

Phosphate buffer pH-7.5	0.01M
PVP-40	5%
Sucrose	20%
2-ME	0.01M
Triton X-100	0.01%

**4) Crushing buffer 4**

Phosphate buffer pH-7.5	0.05M
PVP-40	9.6%
2-ME	0.01M
BSA	0.8%

(a) **Reagent C (alkaline copper solution):** Mixed 50 ml of reagent A and 1 ml of reagent B prior to use.

(b) **Reagent A:** 2% Sodium carbonate in 0.1N Sodium hydroxide.

(c) **Reagent B:** 0.5% Copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in 1% potassium sodium tartrate.

#### **Alkaline copper tartrate :**

a) Dissolved 2.5 g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20g anhydrous sodium sulphate in 80 ml water and made up final volume to 100 ml.

b) Dissolved 50 g copper sulphate in a small volume of distilled water. Added one drop of sulphuric acid and made up final volume to 100 ml.

Mixed 4 ml of B and 96 ml of solution A before use.

#### **Arsenomolybdate Reagent:**

Dissolved 2.5g ammonium molybdate in 45 ml water. Added 2.5 ml sulphuric acid and mixed well. Then added 0.3g disodium hydrogen arsenate dissolved in 25 ml water. Mixed well and incubate at 37°C for 24 to 48 hours.

**PMSF (1M) stock:** Dissolved 17.4 mg PMSF in 1ml ethanol and stored in freeze.

**2X Sample buffer for SDS-PAGE:**

<u>Final concentration</u>	<u>volume taken</u>	<u>Stock</u>
0.125M Tris HCl	0.125 ml	1M
Water	0.175 ml	
4% SDS	0.4 ml	10%
20% Glycerol	0.2 ml	100%
10% 2-ME	0.1 ml	100%
0.004% bromophenol blue	10 mg	

Final volume was made up to 1 ml with distilled water.

**Electrode buffer for SDS-PAGE:**

Tris Buffer	3.0g
Glycine	13.3g
SDS	1g

Dissolved and made the final volume to 1 liter in water.

**Sodium phosphate mono basic buffer (0.2M):** Dissolved 27.8g of sodium phosphate in 200 ml and final volume made up to 1000ml with distilled water.

**Sodium phosphate di-basic buffer (0.2 M):** Dissolved 53.65g of dibasic sodium phosphate in 200 ml distilled water. Final volume made up to 1000 ml.

For pH 6.0, 87.7, ml mono-basic and 12.3 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

For pH 7.0, 39.0 ml mono-basic and 61.0 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

For pH 7.5, 16.0 ml mono-basic and 84.0 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

Acetate buffer:

0.2M solution of acetic acid: 11.55 ml of acetic acid dissolved in 1000 ml of distilled water.

0.2M solution of sodium acetate: 16.4g of sodium acetate dissolved in 200ml-distilled water and made up the final volume 1000 ml.

For pH 5.0, 14.8 ml of acetic acid and 35.2 ml of sodium acetate was mixed and made to final volume to 100 ml to get the buffer of 100 mM.

For pH 5.6, 4.8 ml of acetic acid and 45.2 ml of sodium acetate was mixed and made to final volume to 100 ml to get the buffer of 100 mM.

Tris- citrate starch gel buffer (pH 8.9): Dissolved 9.026 Tris-Cl and 1.051 gm of citric acid and final volume was made up to 1000 ml with distilled water.

Running starch gel buffer (Sodium borate buffer): Dissolved 18.55 boric acid and 4 gm sodium hydroxide and final volume was made up to 1000 ml with distilled water.

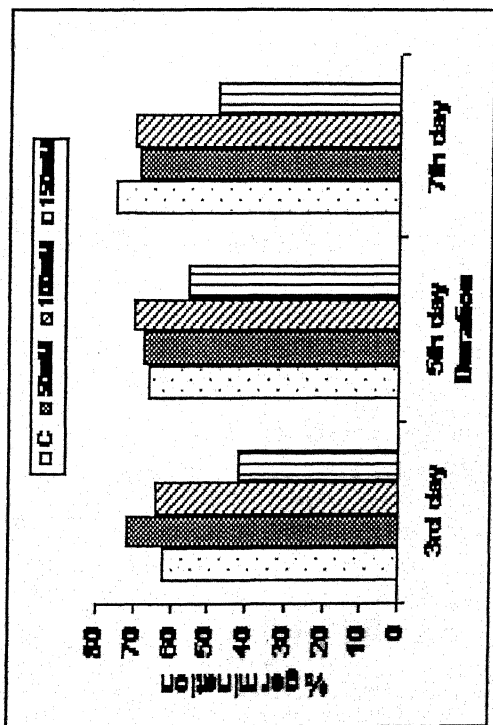
## RESULTS

### Screening of stylo for salinity tolerance:

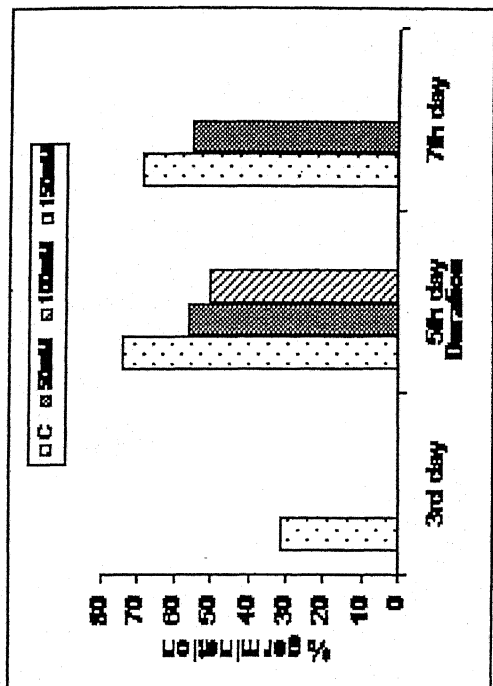
#### Experiment: 1

#### *In-vitro* screening on M. S. media:

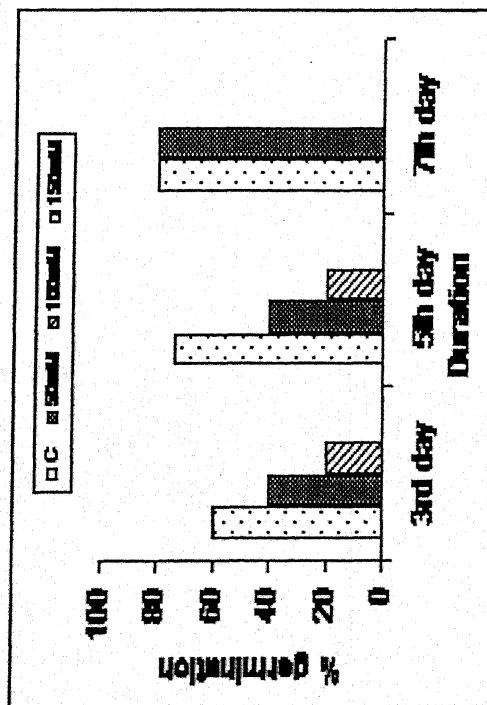
Seeds of seven species comprising six accessions of *S. seabrana* (2523, 104710, 110372, 2539, 2534, 105546-B), ten accessions of *S. scabra* (RRR 94-97, RRR 94-96, RRR 94-86, RRR 94-100, RRR 94-93, Q10042, 36260, cv Seca, 93116, cv Fitzroy), three of *S. hamata* (110123, 110135 and 61670), one each of *S. humilis* (IG110), *S. capitata*, *S. guianensis*, and *S. viscosa* (CPI 33941) were used for screening. De-husked and surface sterilized (0.1% HgCl<sub>2</sub>) mature stylo seeds were thoroughly washed in distilled water and inoculated under aseptic conditions on a solid Murashige Skoog media. Five different levels (control, 50, 100, 150 and 200mM NaCl) of treatment were imposed. Percentage germination and length of seedlings were recorded at three, five and seven days of stress. *In-vitro* screening of 23 accessions, indicated variations in percentage in seed germination under different levels of salinity (Fig 4 A – E). The NaCl concentration, at which seedlings showed no response and turned brown, was treated as inhibitory concentration. At third day of germination *S. seabrana* showed increase in germination as compared to control. *S. seabrana* showed 70% germination at 50mM of NaCl stress, whereas at 100mM germination was decreased to 60%. It was greatly reduced to 40% at 150mM salt stress. At fifth day of stress seed germination in 50mM NaCl was 60%, but at 100mM it was higher i.e., 65%. At 150mM NaCl stress germination was somewhat better and it reached up to 70%. At seventh day germination was 70% even in control, but at 50 and 100mM, it was reduced to 60% and 65% respectively. Germination was reduced to 50% at 150mM. *S. scabra* showed germination in only control plates on third day and that was 70%. In 50mM NaCl stress germination was 50% and it was further reduced to 40% at the level of 100mM salt stress (Fig 5A and B). At seventh day germination at 50mM was 50% to that of fifth day. In *S. scabra* browning of seeds in the presence of 150mM of NaCl stress was observed. In *S. viscosa* 60% germination was



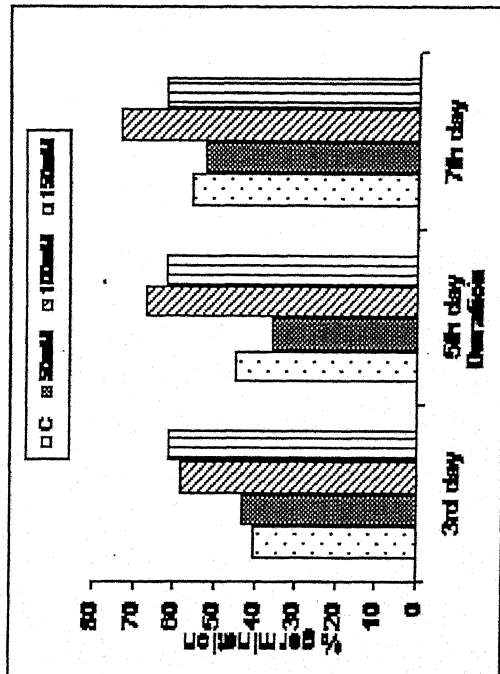
*S. seabrana*



*S. scabra*

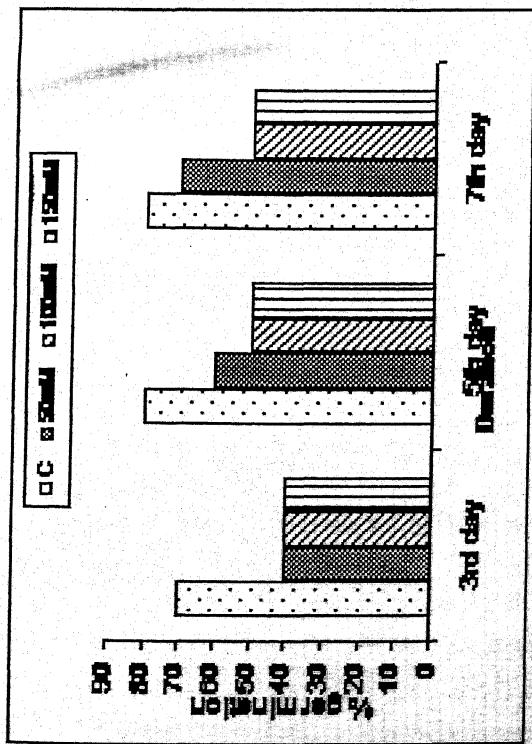


*S. viscosa*

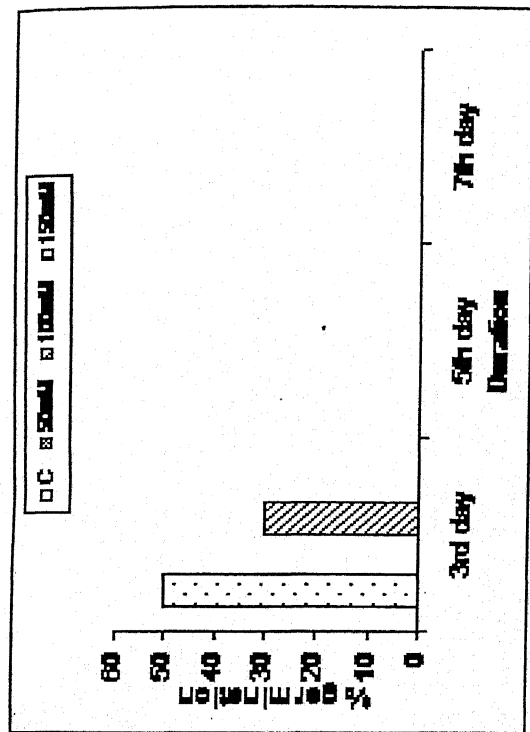


*S. hamata*

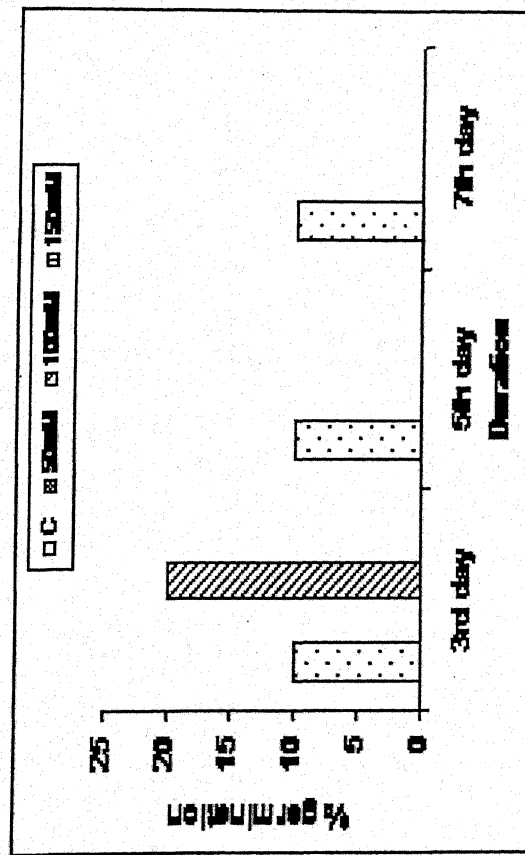
Fig 5A: Response of NaCl on seed germination including survival of seedlings with time under *in-vitro* condition



*S. humilis*



*S. guianensis*



*S. capitata*

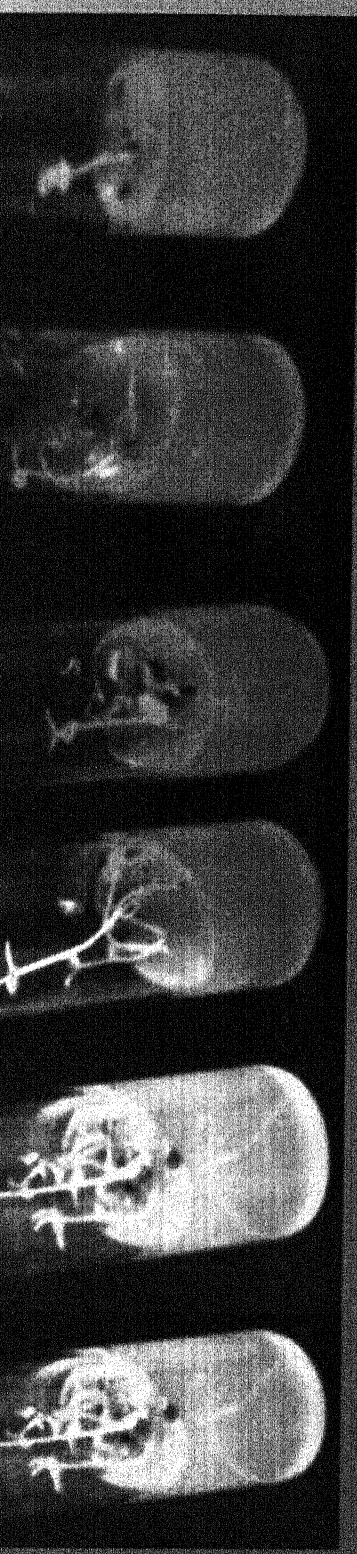
Fig 5B: Response of NaCl on seed germination and survival of seedlings under *in-vitro* condition



44: *In vitro* seedling germination vigour of *S. scabra* accessions L-R. (RRR94-97, Flizoy, RRR94-96, Q10042, RRR94-86, 36260, RRR94-100, RRR94-93, CPI 40292, Secca) C. Control; T1. 50mM; T2. 100mM; T3. 150mM.



4B: *In vitro* seedling germination vigour of *S. seabrana* accessions L-R. (CPI2523, CPI 104710, CPI 2539, CPI 2534, CPI 105546B) C. Control; T1. 50mM; T2. 100mM; T3. 150mM



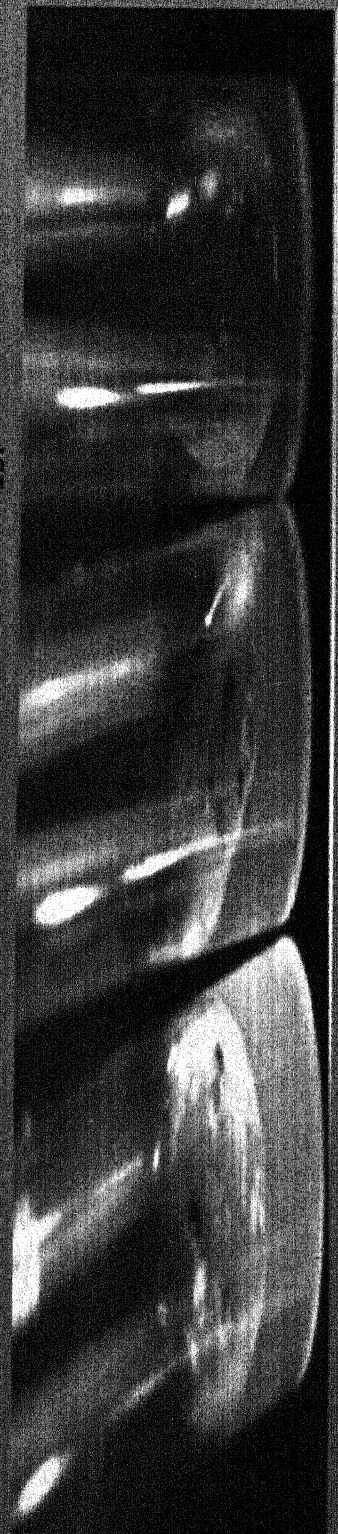
C.

T1.



T2.

T3.



T4.

Fig 4C: *In vitro* seedling germination vigour of *S. hamata* accessions (L-R) CPI 110123, CPI 110135, CPI 61670, C. Control; T1. 50mM; T2. 100mM; T3. 150mM.

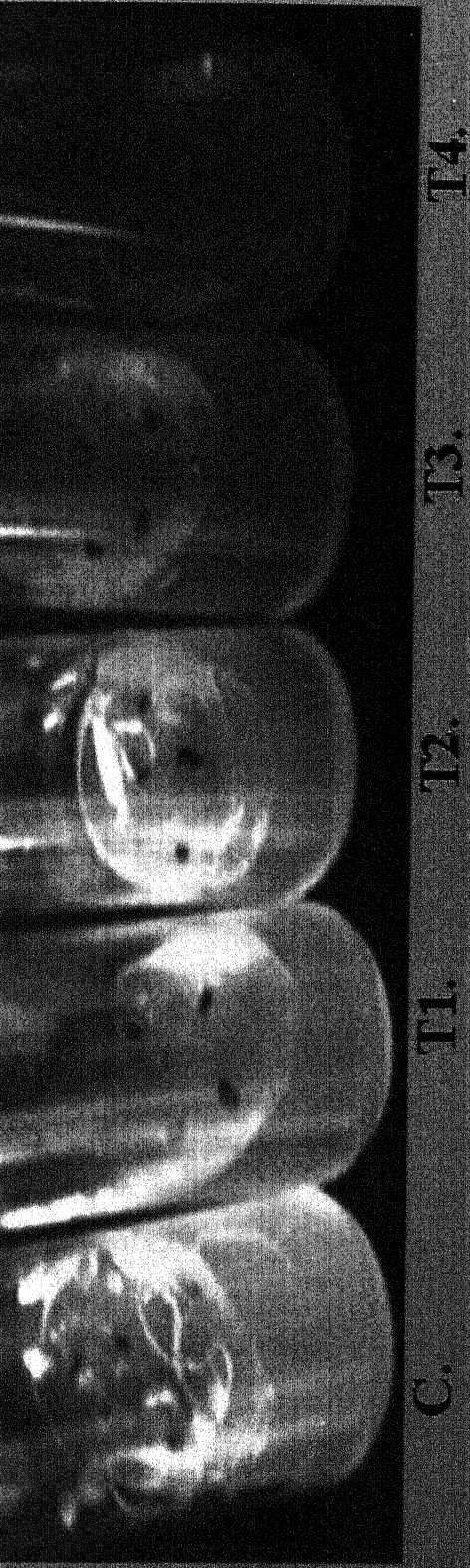
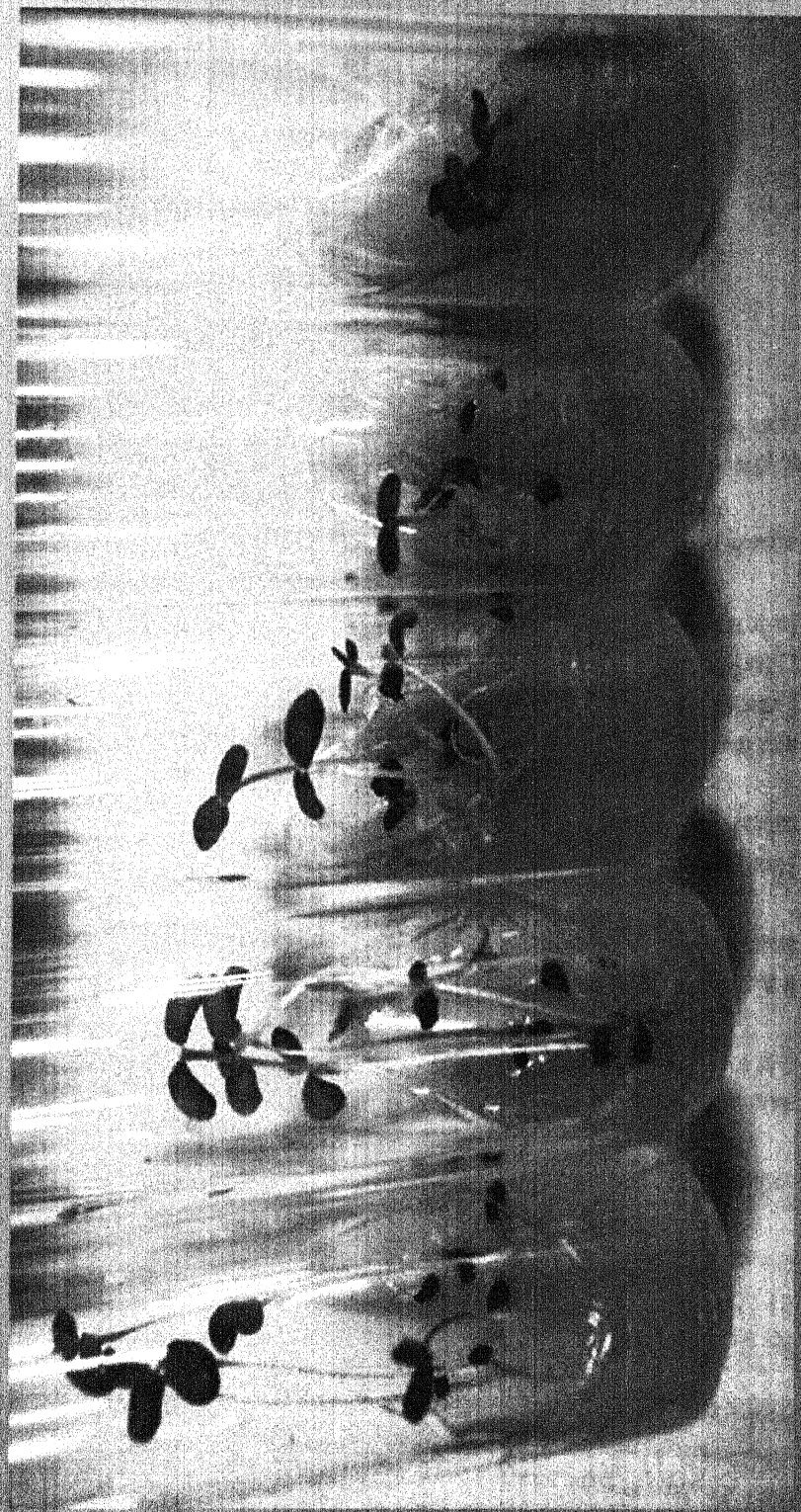


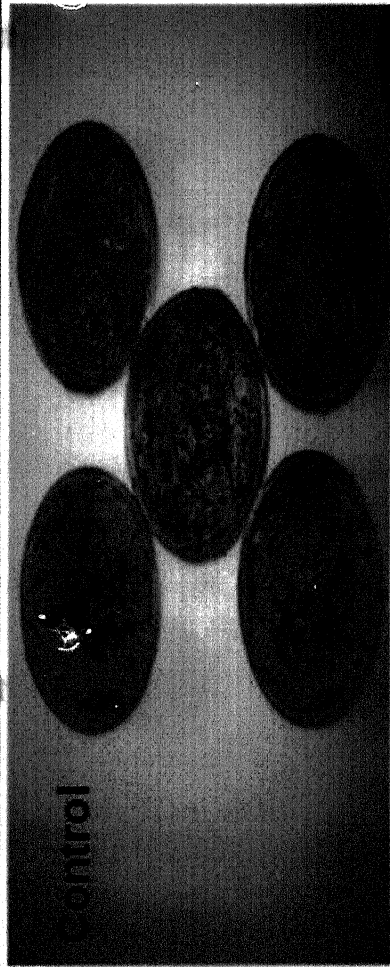
Fig 4D: *In vitro* seedling germination vigour of *S. guianensis* (Upper), *S. viscosa* (Lower). C. Control; T1. 50mM; T2. 100mM; T3. 150mM; T4. 200mM.



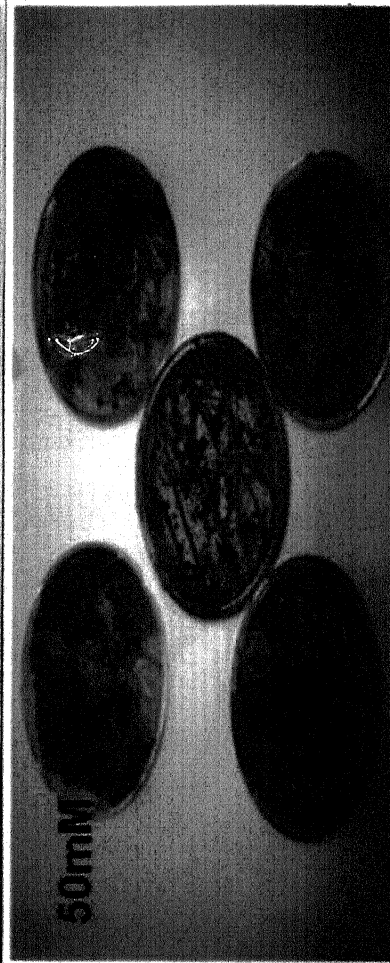
C. T1. T2. T3. T4.

Fig 4E: *In vitro* seedling germination vigour of *S. humilis*. C. Control; T1. 50mM; T2. 100mM; T3. 150mM; T4. 200mM.

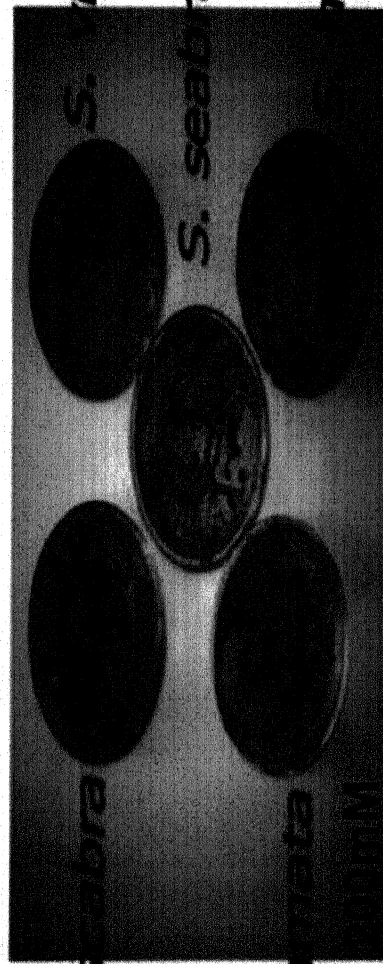
observed in control plates on third day. At 50mM germination was 40% and it was reduced to 15% when the stress level was 100mM. At fifth day of stress *S. viscosa* showed same pattern as it was found in *S. scabra*. Control plates showed 70% germination and it was 40% at 50mM level of salt stress. In 100mM of salt stress *S. viscosa* showed decrease in germination (10%). At seventh day, germination in control was higher than third and fifth day of salt stress, and it was up to 80%. Germination percentage of *S. viscosa* at 50mM of NaCl stress was same as it was in control i.e., 80%. *S. hamata* showed same pattern as indicated by *S. seabrana* but it has grown better even at 150mM of NaCl stress. At third day of stress, seed germination was 45% at 50 mM and it was more than control plates (40%). Sixty percent germination of seeds was observed in the 100mM of NaCl stress. Germination was linearly increased to 65 % at 150mM level of salt stress. At fifth day of stress, germination was reduced to 30% at 50mM of salt stress as compared to control (45%). At 100mM stress germination was increased up to 70% in *S. hamata*. *S. hamata* showed maximum germination (60 %) at 150mM of salt stress. At seventh day of stress, germination was 60 % at 50mM of salt stress and it was increased maximum to 70% at 100mM level of salt stress. At 150mM of NaCl stress, germination was reduced to 60%. In *S. humilis* germination percentage was 70% in control plates. At 50, 100 and 150mM level of salt stress, germination was reduced to 40% in *S. humilis*. *S. guianensis* showed much germination retardation under NaCl stress as compared to other species. Germination was 10% at third, fifth and seventh day of salt stress. At 50mM stress, *S. guianensis* showed no germination but at 100mM it was increased up to 20%. At 150mM of NaCl stress no germination was observed in *S. guianensis* plates. At fifth and seventh day of salt stress no germination was observed in *S. capitata* as *S. guianensis*, fifty percent germination was showed in control plates. At 50mM NaCl stress, there was no germination but at third day under 100mM stress level, 30% germination was observed. Further growth of seedlings was much inhibited in higher stress levels. *S. hamata*, *S. humilis* and *S. seabrana* performed better in terms of germination at 50, 100 and 150mM of NaCl over other species. Seedling length was also observed better in these three species from other species, whereas *S. capitata* and *S. guianensis* showed very high retardation of growth even in control plates.



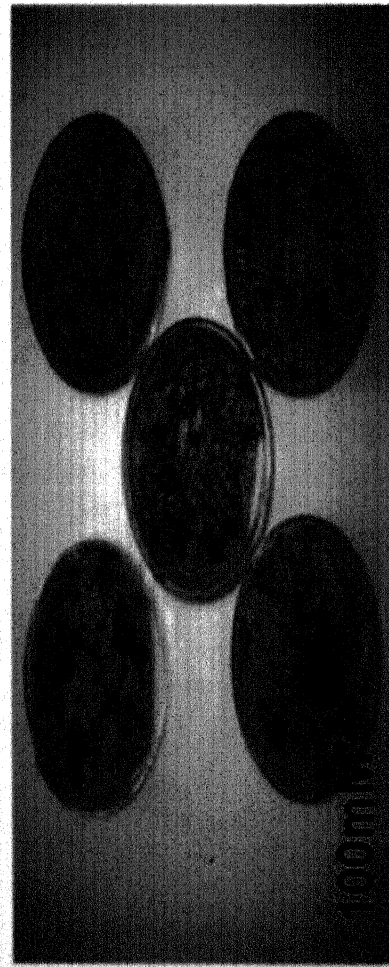
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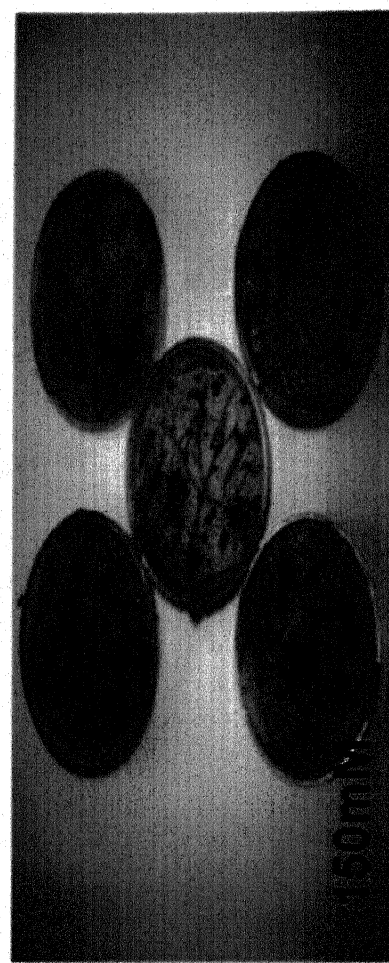
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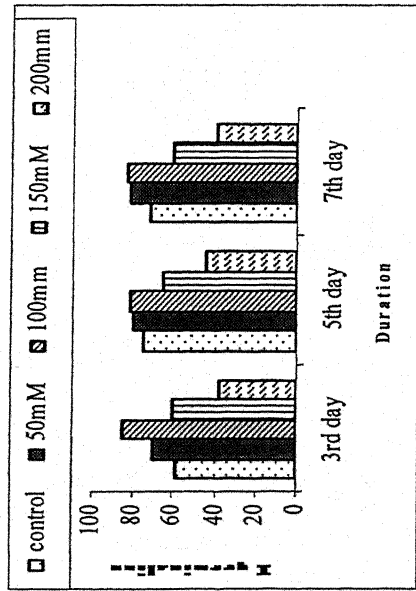


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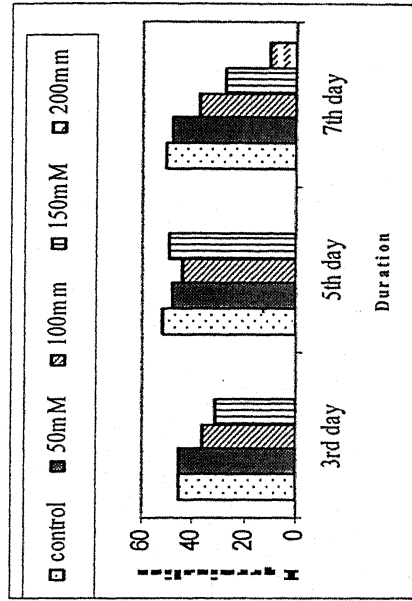


4

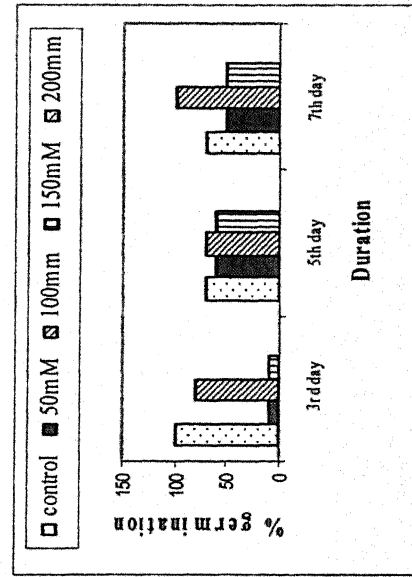
Fig 6: Response of NaCl on seed germination in petri plates



*S. Seabrana*



*S. scabra*



*S. viscosa*

Fig 7A: Response of NaCl on seed germination with survival with time of seedlings in petri plates

## **Experiment 2:**

### **Screening on petri plates at room temperature:**

To study the performance of seeds of best five *Stylosanthes* species screening was performed further on petri plates with moist filter paper with water (control), 50mM, 100mM and 150mM NaCl for different stress conditions. At third day of stress germination was 60% in *S. seabrana*. It was increased to 70% at 50mM of NaCl stress and at 100mM it was further increased up to 90%. At 150mM level of stress germination was reduced to 60% and with increasing salinity (200mM) germination was reduced to 30%. At fifth day, germination in control, 50mM and 100mM was 70, 80 and 90% respectively however at 150 mm of salt stress it was 60% and at 200mM it was 40%. Control plates of *S. seabrana* at seventh day of seed germination showed 70% germination, but it was increased to 90% in 50 and 100mM of stress. Fifty percent germination was observed at 150mM of stress. At 200mM level of salt stress, germination was reduced to 40%. *S. scabra* showed 50% germination in control as well as at 50mM level of NaCl stress at third day of stress. It was decreased to 30% and 25% at 100 and 150mM of NaCl respectively (Fig. 6). Germination percentage was better at fifth day of salt stress. It was observed 50% in 50mM of NaCl stress and 40% in 100mM of salt stress (Fig 7A and B). Fifty percent germination was shown by *S. scabra* at fifth day of stress at 150mM NaCl. At seventh day of stress germination was 50% at 50mM and it was decreased to 40% at 100mM level of NaCl stress. At 150mM germination was 30% and at 200mM it was observed only 10%. At higher concentrations along with germination the survival of the seedlings was also taken into account and thus decrease in seedling germination was reported.

*S. viscosa* was much affected by NaCl stress at 50mM and 150mM. However at 100mM germination was 80%. Control plates showed less germination at fifth day of stress in *S. viscosa*. At 50, 100 and 150mM of NaCl stress, germination was 60, 70 and 80% respectively. It was increased to 100% at 100mM salt stress, and at 150mM of salt stress 50% germination was observed in *S. viscosa*. Eighty percent germination was observed in *S. hamata* in control plates at third day of seed germination. It was reduced to

70% at 50mM level of salt stress. At 100mM of NaCl stress germination was 60%. Increased germination (90%) was found at 150mM of stress in *S. hamata*. Germination was further reduced to 70% at 200mM of salt stress. At fifth day of NaCl stress, 80% germination was observed in 50mM of stress. At 100mM of salt stress, germination was reduced to 60% in *S. hamata*. At 150mM and 200mM germination was further increased up to 80%. At seventh day of salt stress in *S. hamata* germination was 80% at 50mM NaCl, however it was 70% in control plates. Sixty to seventy percent germination was shown in 100 and 150mM of NaCl stress respectively. At 200mM of salt stress *S. hamata* showed decrease in germination (40%).

In *S. humilis*, at 50mM germination was 80% and it was decreased to 70% at 100mM of salt stress. At 150mM and 200mM NaCl level, germination was 60% and 50% respectively at third day of stress. At fifth day, germination was 70% at 50mM of NaCl. Germination was decreased to 60% at 100mM level of NaCl stress. Increased germination (100%) was observed in *S. humilis*, at 150mM of stress. At 200mM stress level germination was reduced to 60% in *S. humilis*. At seventh day of stress *S. humilis* showed 70% germination at 50mM of stress. In control germination was also 70%. Germination was increased to 80% at 100mM of salt stress. At 150mM germination was decreased to 60% and it was increased to 70% at 200mM level of NaCl stress. *S. capitata* and *S. guianensis* showed no germination in NaCl stress conditions, while in control plates germination was 10 % in *S. guianensis* and 30% in *S. capitata*.

### **Experiment 3:**

#### **Single salt experiment:**

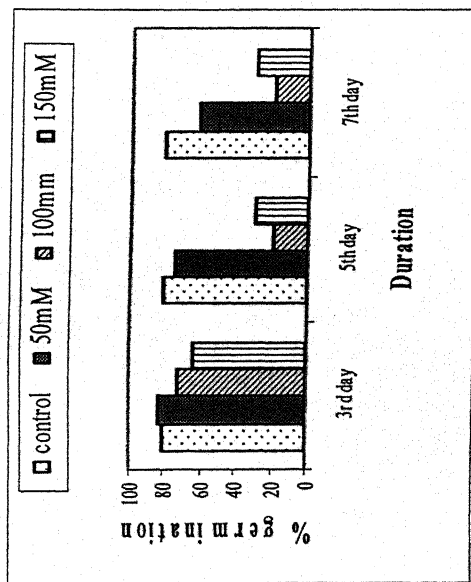
In this experiment seeds of five species of *Stylosanthes* namely *S. seabrana*, *S. scabra*, *S. viscosa*, *S. hamata* and *S. humilis* were grown in NaCl mixed soil in glass house for the study of salt tolerance in *Stylosanthes* species. Pots filled with soils containing 50, 100 and 150mM NaCl level were used in this study. A small pipe in each pot was placed for proper reaching of salts as and when pots were irrigated. Seeds were placed in soils for germination. When the soil of pots looked dry water was given to pots once in a week

with the help of pipe so as to reach to the bottom of the pots, In *S. seabrana* at third day of salt stress germination was 80% in 50mM, as well as in control plates. It was reduced to 70% at 100mM and 60% at 150mM. Germination was 85% in control on fifth day and 80% at 50mM NaCl. It was highly reduced to 20% at 100mM whereas 40% at 150mM level of salt stress (Fig. 8A and B). *S. seabrana* showed 70% germination at 50mM NaCl. It was reduced to 20% at 100mM salt stress and then increased to 40% at 150mM level of salt stress level. Linear decreased in germination with increasing salinity was observed in *S. scabra* at all levels of salt stress. At third day of stress, at 50 and 100mM level, germination was 40 and 20%. Germination in control was also observed low (50%). At 50, 100 and 150mM levels of salt stress germination was 30%. *S. viscosa* showed inhibition in germination. Germination was 80% in control plates of third, fifth and seventh day of germination, whereas at 50mM germination was 10% at third, fifth and seventh day of salt stress. *S. hamata* showed 70% germination in control plates at third, fifth and seventh day of germination. Which decreased to 30% at third day in 50mM of stress. It was increased to 80% at 100mM of salt stress and further decreased to 30% at 150mM of stress. At fifth day of stress germination was 50% at 50mM and increased to 60% at 100mM stress level. This further increased to 70% germination at 150mM level of NaCl in soil. At seventh day of stress, 40% seeds germinated at 50mM level of salt, and it was increased to 50% at 100mM level. Further decreased in seed germination was observed (30%) in *S. hamata*. In control and in 50mM level of NaCl stress germination was 70 and 60% respectively in *S. humilis* at third, fifth and seventh day of stress. At 100 and 150mM, germination was reduced to 10% during different days of NaCl stress. *S. guianensis* and *S. capitata* showed 20% germination in control plates at third, fifth and seventh, days of NaCl stress. At third day and fifth day, however, germination was 10% in both species at 50mM of salt stress.

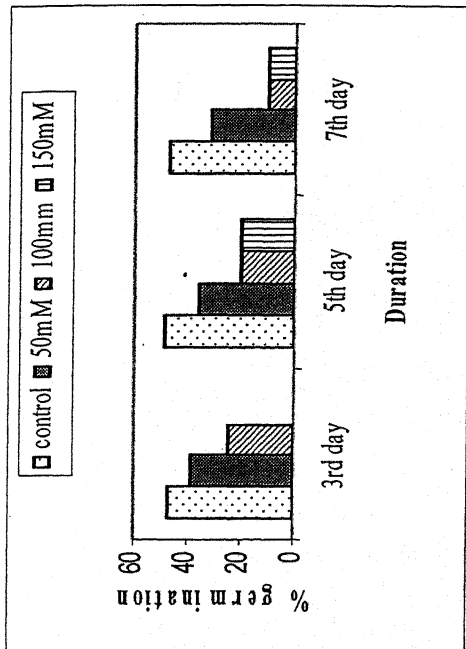
#### **Experiment number 4:**

##### **Mixed salt experiment:**

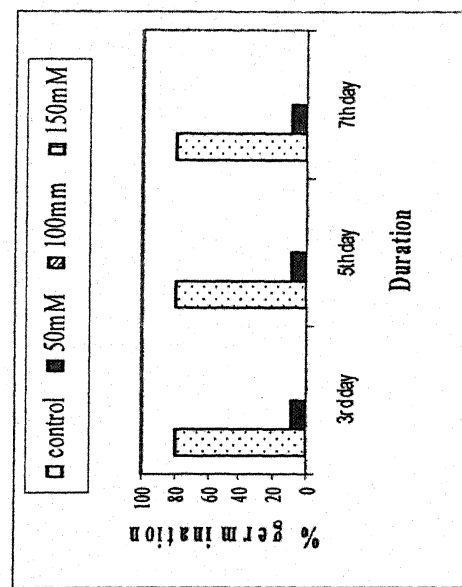
In this treatment, seeds of *Stylosanthes* was germinated in pots filled with soil of mixed salt. By mixing different salts 1, 1.5, 2, 2.5, 4, 6 and 8 EC were maintained in soil. When



*S. seabrana*

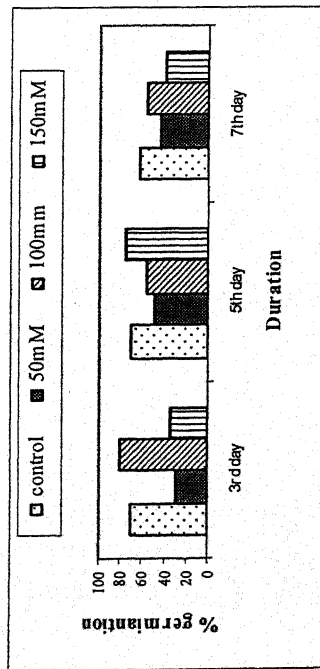


*S. scabra*

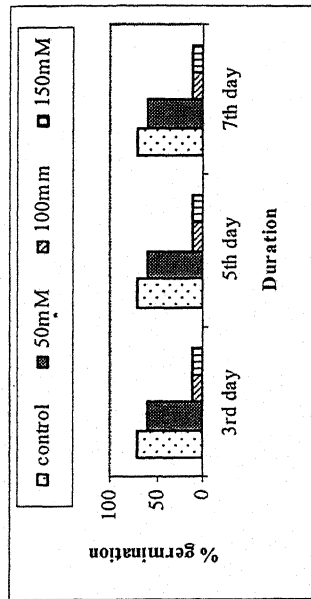


*S. viscosa*

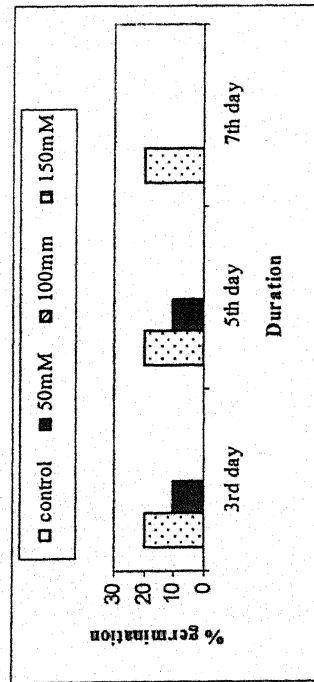
Fig 8A: Response of NaCl on seed germination and survival of seedlings in soil



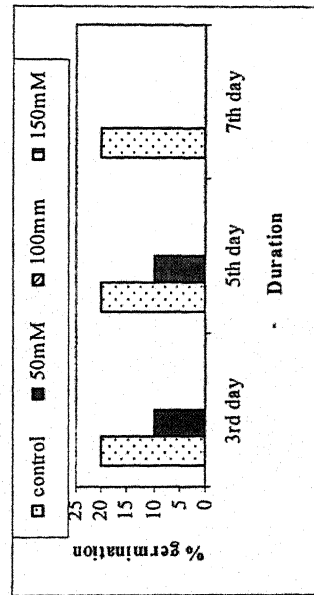
*S. hamata*



*S. humilis*



*S. guianensis*



*S. capitata*

Fig 8B: Response of NaCl on seed germination and survival of seedlings in soil

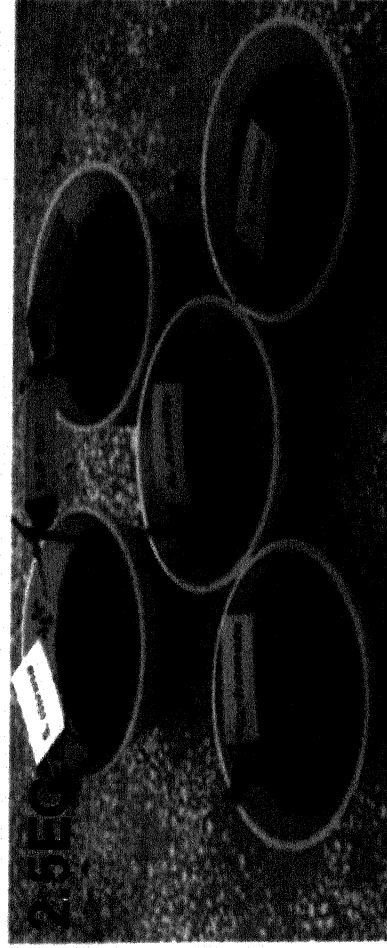
the soil of pots looked dry from upper surface water was given once in a week with the help of thin pipe so as to reach to the bottom of the pots. Seeds of *Stylosanthes* showed maximum inhibitory effect with mixed salt treatment. *S. seabrana* showed much retardation in mixed soil as compared to single salt experiment. In the first week of experiment germination was better in control pots. At third day of stress 15% germination was observed in control pots of *S. seabrana*, it was reduced to 10% at 1EC stress level. At 1.5EC germination was increased to 20%. At 2 and 2.5EC germination was 10 and 5% respectively (Fig. 9). At fifth and seventh day of stress germination was 30% and 40% was in control and it was decreased linearly with increasing level of salt stress. *S. scabra* showed highest germination in control pots at third, fifth and seventh days of stress. At third day it was decreased with the increasing salt level. At fifth day of stress in 1 and 1.5EC germination was lower than 2 and 2.5EC. *S. scabra* showed decreased germination percentage with the onset of stress levels, i.e., 1EC. Germination was 18% in control pots of *S. viscosa* at third day of stress. It was reduced to 9% at stress level of 1EC and increased to 18% at 1.5EC salt level. At 2 and 2.5EC germination was 14 and 5% respectively. At fifth day of stress germination was 20% in control pots but was decreased to 10% at 1EC stress level. At 1.5EC germination was 15% and it was linearly decreased with increasing salt stress. At seventh day in 1 and 2.5EC germination was low (5%) but in 1.5EC and 2EC it was 15%. Germination in *S. hamata* was 15% in control and was decreased to 8% at 1EC. Increased germination was observed at 1.5 and 2EC stress level. At 2.5EC germination was decreased to 5%. At fifth day of stress 25% germination was observed in control pots, it was decreased to 20% in 1EC. *S. hamata* showed 15 and 10% germination in 1.5 and 2EC. At seventh day in control and 1.5EC germination was higher than it was in 1, 2.0 and 2.5EC. Germination in *S. humilis* was 10% in control pots, whereas 5% in 1EC. It was increased to 10% at 1.5EC and further decreased to 5% at 2.0 and 2.5EC. At fifth day of stress germination was 10% in 1EC and it was decreased to 5% at 1.5EC. In 2EC stress level germination was increased to 10% which further decreased to 5% in 2.5EC. At seventh day germination was 20% in control pots, while it was 10% in 1EC. Germination was decreased to 5% in 1.5, 2 and 2.5EC stress level. In *S. guianensis* and *S. capitata* germination was observed 15% and 20% respectively in control pots of these species. There was no germination observed in both



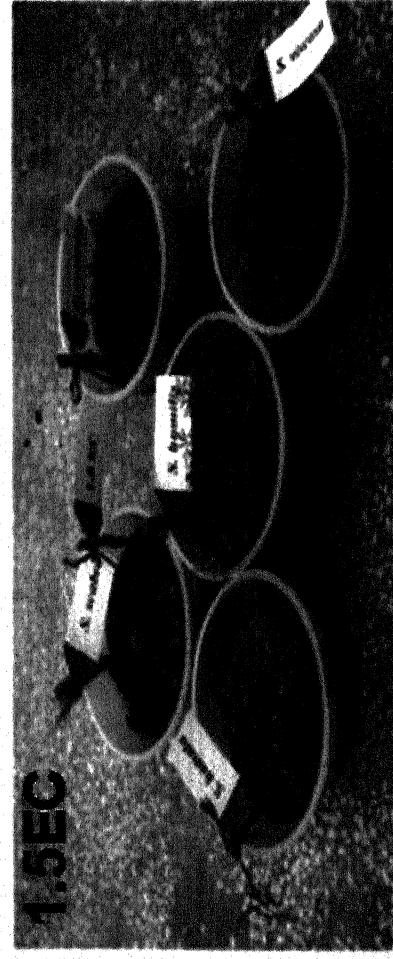
1



2



5



3



4

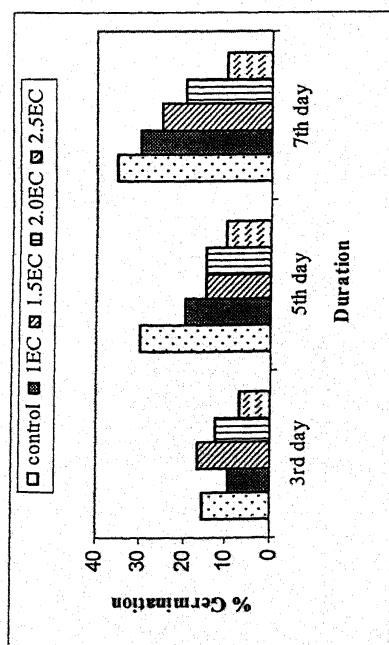
Fig 9: Response of NaCl on seed germination in soil

the species in salt stress treated soil. Germination in *S. seabrana* reduced to 30% and 20% and 30% at 4 and 6EC of salt stress respectively at third day of stress. At 8EC germination was reduced to 10% in *S. seabrana*. It was reduced to 20% at 4 and 6EC. At 8EC germination was reduced to 10%. *S. scabra* showed 15% germination at 4EC. There was no germination at 6 and 8EC in *S. scabra* at third day of stress. Gradually decreased germination was observed in *S. scabra* at fifth day of stress. Germination was decreased to 30, 20 and 10% at 2, 4 and 6EC of salt stress. At seventh day germination was 20 and 10% at 4 and 6EC. *S. viscosa* showed 30% at 4EC and 80% seed germination at fifth day of stress at 2EC, and it was drastically reduced to 20% at 4EC salt stress. At seventh day of salt stress germination was increased to 60% as compared to control where it was 40%. It was reduced to 10% at 4EC level of salt stress. *S. hamata* showed not much positive response as it was shown in other experiments but it was germinated well even at 8EC level of salt stress. Germination was 20% in control of third day, and it was increased to 30% at 2EC salinity level. At 4EC germination was reduced to 15% and it increased to 20% again at 6EC salinity level. Germination was reduced to 10% at 8EC salinity level. At fifth day of stress, germination was increased to 30% at 2EC level compared to control (25%), and at 4 and 6EC germination was 20% (Fig 10 A and B). Germination was reduced to 15% at 8EC salinity level. *S. hamata* showed better performance at seventh day of stress in control as well as in stress pots. Germination was 40% at 2EC and it was reduced to 20% at 4EC. It was increased to 25% at 6EC and at 8EC germination was 10%. In *S. humilis*, germination 20% at 4EC at third day of stress, increased to 30% at third day in 6EC level of salinity. Germination was 20% at 2EC which was increased to 30% in 4EC level of salinity at fifth day of stress. Germination was decreased to 20% in 6EC level of soil. At seventh day germination was 20% at 4EC. At 6EC germination was increased to 40% at seventh day of salt stress.

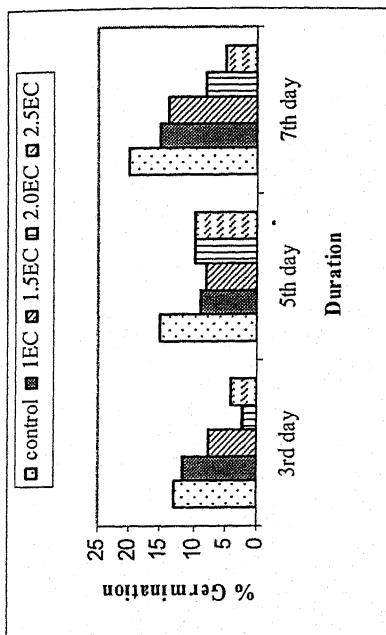
#### Experiment 5:

##### **Isozyme analysis in seedlings:**

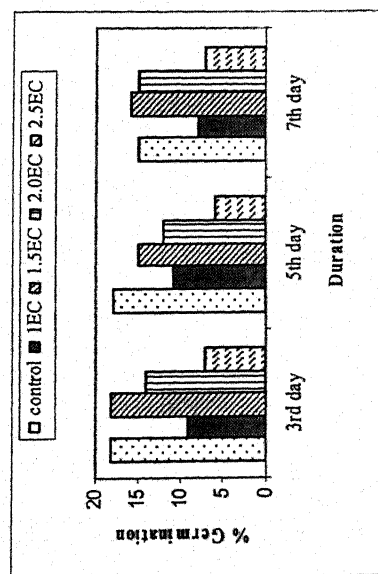
Seedlings of five species of *Stylosanthes* namely *S. seabrana*, *S. scabra*, *S. viscosa*, *S. hamata* and *S. humilis* were grown on filter paper fixed petri plates. Before placing the seeds, Petri plates were treated with fungicide. Filter paper bed was soaked with water



*S. seabrana*

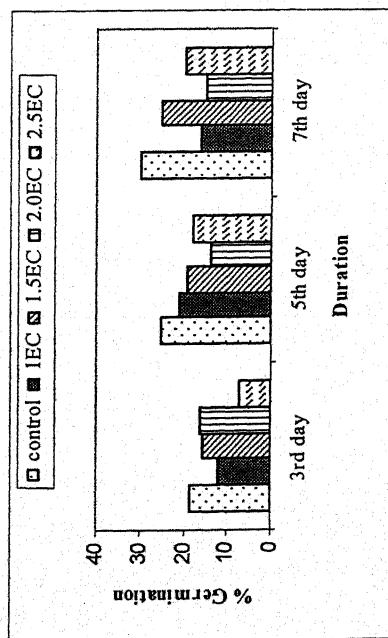


*S. scabra*

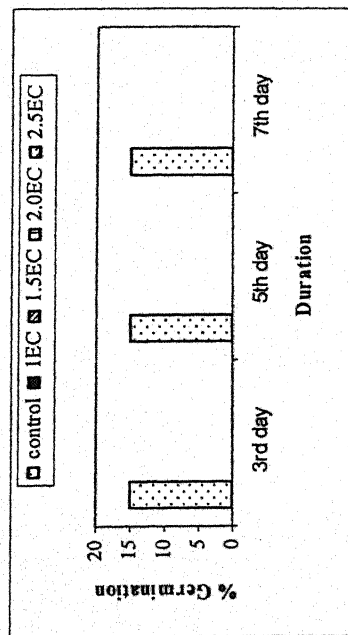


*S. viscosa*

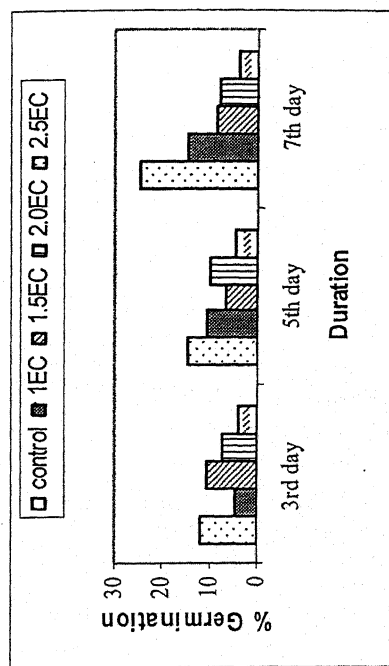
Fig 10A: Response of mixed salts on seed germination including survival of seedlings in soil



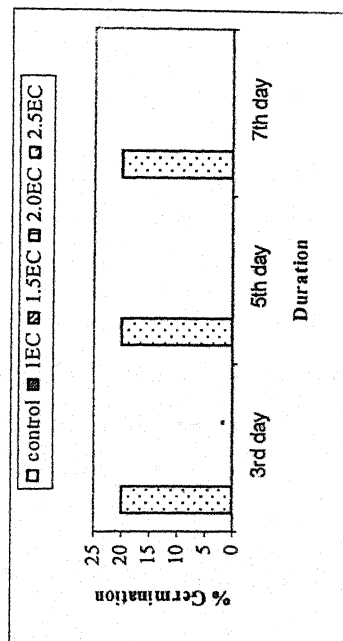
### *S. hamata*



### *S. guianensis*



### *S. humilis*



### *S. capitata*

Fig 10B: Response of mixed salts on seed germination including survival of seedlings in soil

(control), 50mM, 100mM 150mM NaCl as different stress conditions. Isozyme analysis of 14 enzyme system was carried out using 12% acrylamide and 14% starch gel. All the fourteen enzyme systems showed good resolution, staining and banding pattern was reproducible for each species analyzed. On the basis of gel patterns zymogram of enzymes was developed. Relative mobility ( $R_m$ ) of each band was calculated as the ratio of distance traveled by the band to the distance traveled by the tracking dye. Bands were numbered on the basis of increasing  $R_m$  values. Loci and alleles were subsequently numbered and lettered respectively. The isozyme patterns were defined by taking into account the number and position of bands from top of the gel.

### **Esterase (EST)**

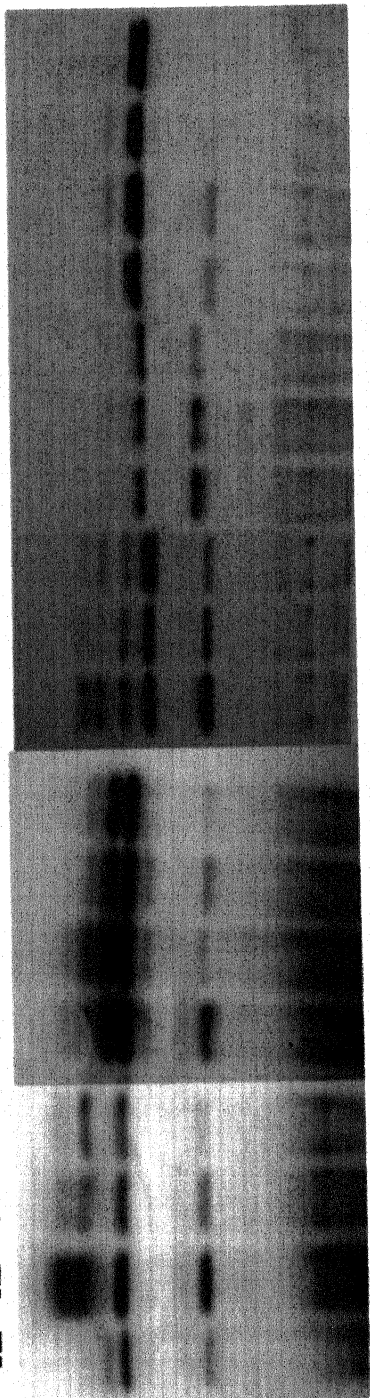
Non denaturing PAGE revealed 13-esterase isozyme bands and were denoted as EST-1 to EST-13. In *S. seabrana* 4 isoforms namely EST-2, 10, 11 and 12 were observed. Quantitative change in isoform 2, 10 and 11 was observed in stress condition, however the intensity of isoform 12 increased at 100 mM NaCl concentration. In *S. scabra* 6 isoforms viz., EST-1, 2, 6, 10, 11 and 12 were observed. Both qualitative and quantitative changes in isoforms were observed when level of salinity was increased. At 50 and 100 mM, a new isoform namely EST-5 was observed. Increase in intensity of EST-6 was also observed at this concentration. In case of *S. viscosa* both increase and decrease in intensity of isoforms were observed. Intensity of isoforms 11, 12 and 13 were increased whereas EST-9 decreased under higher level of salinity (Fig. 11B and 11F). Isoform-9 appeared as a new isoform in case of *S. hamata* at 150 mM level of salinity. Intensity of isoform 1, 2, 5, 8 and 10 increased when level of NaCl increased from control to 200 mM. Of the total 9 isoforms as observed in *S. hamata*, isoform-12 did not show any change in quantity after imposition of salinity stress. In case of *S. humilis*, eight isoform were observed. At 150 mM increase in intensity of isoforms were observed in comparison to control.

### **Superoxide dismutase (SOD)**

In total eight isoforms of SOD was observed and were numbered as SOD-1 to SOD-8. At lower level of salinity stress no quantitative change was observed in *S. seabrana*,

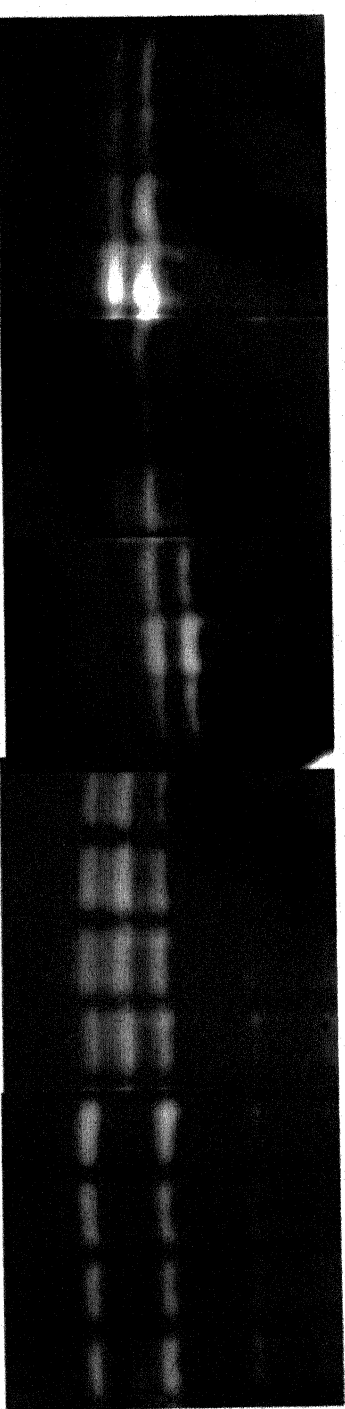
C T1 T2 T3 C T1 T2 C T1 T2 C T1 T2 T3 C T1 T2 T3

*S. scabrana*      *S. scabra*      *S. viscosa*      *S. hamata*      *S. humilis*



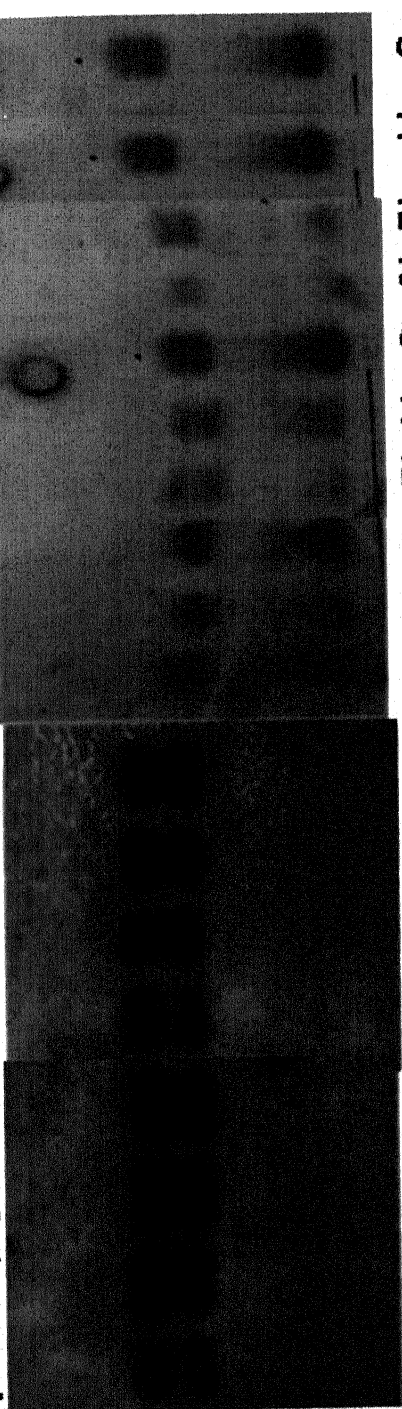
EST

C T1 T2 T3 C T1 T2 C T1 T2 C T1 T2 T3 C T1 T2 T3



SOD

C T1 T2 T3 C T1 T2 C T1 T2 C T1 T2 T3 C T1 T2 T3



GOT

Fig 11B: Isozyme patterns of esterase, super oxide dismutase, glutamate oxalo transaminase in five different species of stylo under control (C), 50mM (T1), 100 mM (T2), 150 mM (T3)

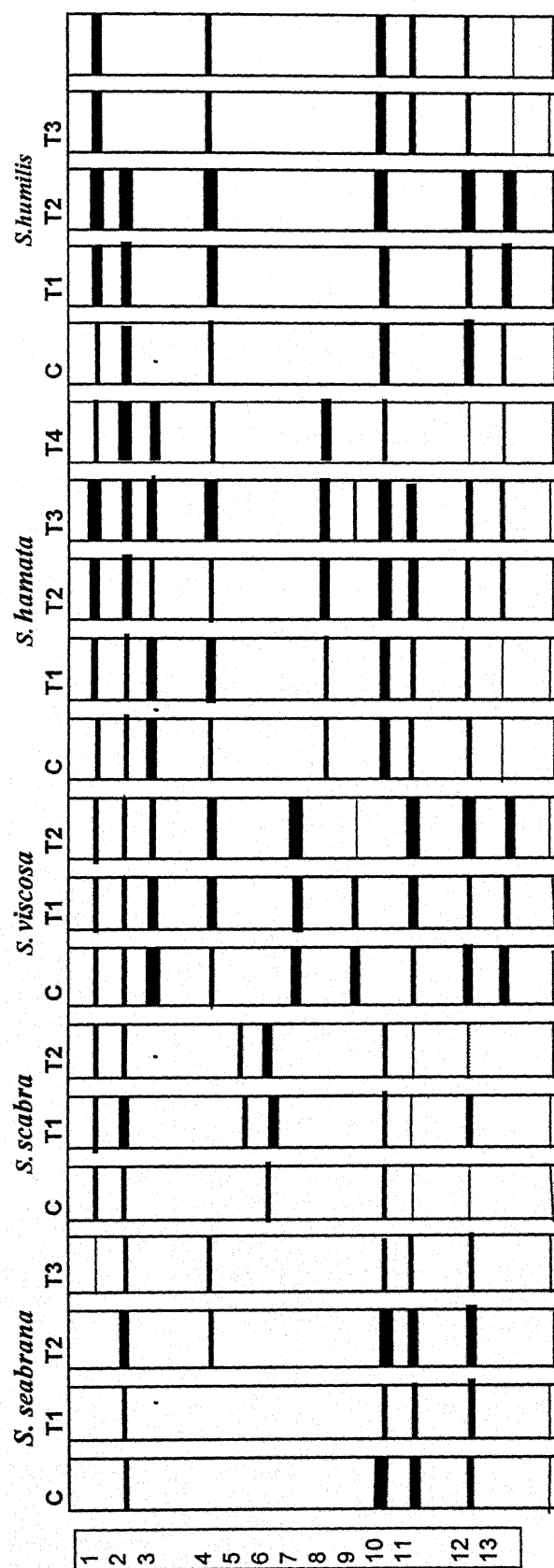


Fig.11B. Zymogram of EST in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

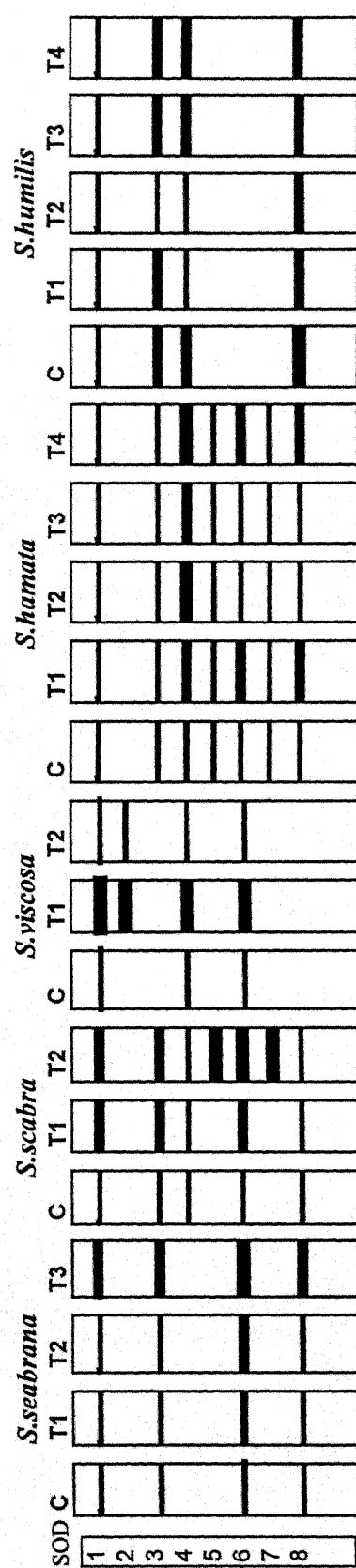


Fig.11B. Zymogram of SOD in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

however intensity of SOD-6 and 8 increased at 150mM level of salinity. In *S. scabra*, two new isoforms viz., SOD-5 and SOD-7 appeared in 100mM stress. In *S. viscosa* SOD-2 isoform appeared at 50mM stress and intensity of SOD-4 isoform increased at 50mM stress. Out of eight isoforms observed in *S. hamata*, five isoforms remained unchanged whereas SOD-6 intensified at 200mM stress however SOD-8 isoform disappeared at this level. In *S. humilis* no qualitative and quantitative changes were observed (Fig 11B and 11G).

#### **Catalase (CAT)**

It revealed only one isoform in all species of *Stylosanthes* and qualitative change was observed in isoform. Intensity of isoform-1 was increased after imposition of stress in some species of *Stylosanthes*. *S. seabrana* showed increase in intensity of this isoform which decreased at 100mM stress. *S. scabra* and *S. viscosa* showed similar patterns of decrease in isoform intensity whereas, *S. hamata* and *S. humilis* showed increase in intensity of catalase isoform (Fig. 11C and 11J).

#### **Glutamate oxaloacetate transferase (GOT)**

In total 4 isoforms namely GOT-1 to GOT-4 was observed. Only quantitative change was observed. In *S. seabrana* intensity of GOT-3 and GOT-4 isoforms decreased at 150mM stress, whereas intensity of only GOT-1 decreased at 100mM stress level. Intensity of GOT-1, 3 and 4 decreased in *S. scabra* after exposure to stress. In *S. viscosa*, isoforms 1 and 4 remains unchanged in salt stress whereas intensity of GOT-3 decreased at 50mM. Intensity of GOT-1 and GOT-2 decreased at 100mM and 150mM of stress levels respectively in both *S. hamata* and *S. humilis* species (Fig. 11B and 11K).

#### **Peroxidase (PRX)**

In total 4 isoforms of PRX was observed. In *S. seabrana* PRX-2 showed no qualitative and quantitative change whereas intensity of PRX-3 and 4 increased at 50mM stress level. PRX-4 isoform disappeared at stress level of 150mM. No change was observed in *S. scabra*, whereas in *S. viscosa*, PRX-4 isoform appeared as a new isoform at 50mM stress and PRX-1 disappeared at 100mM stress level. Two isoforms remains unchanged

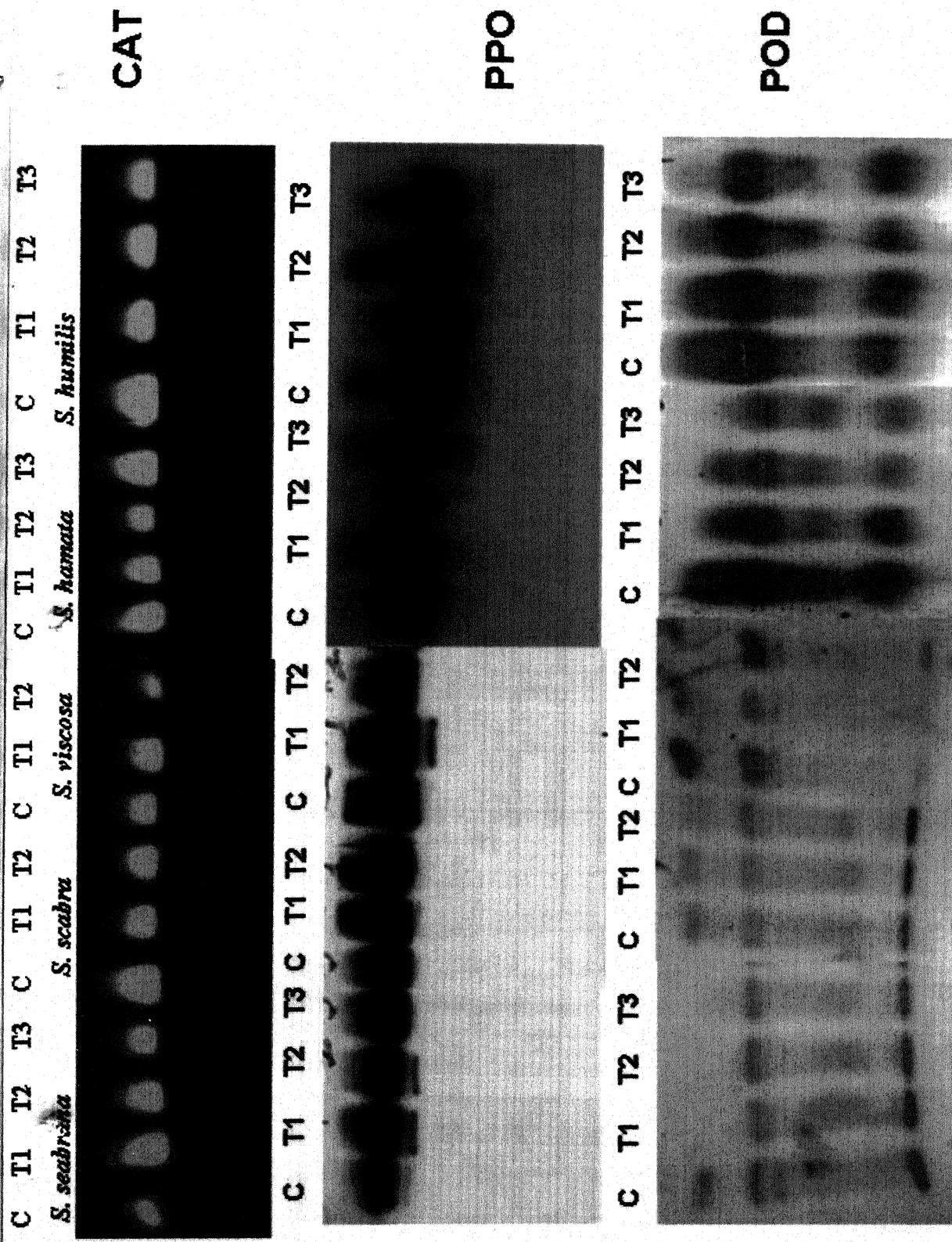
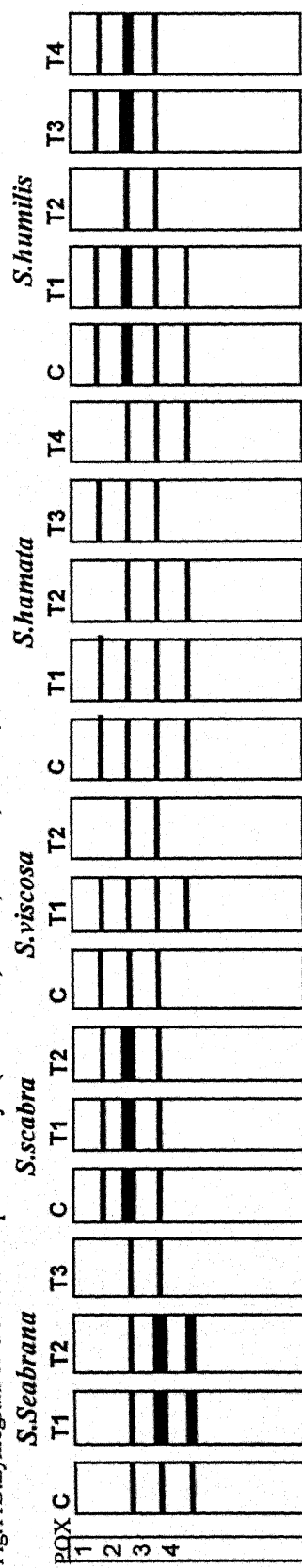
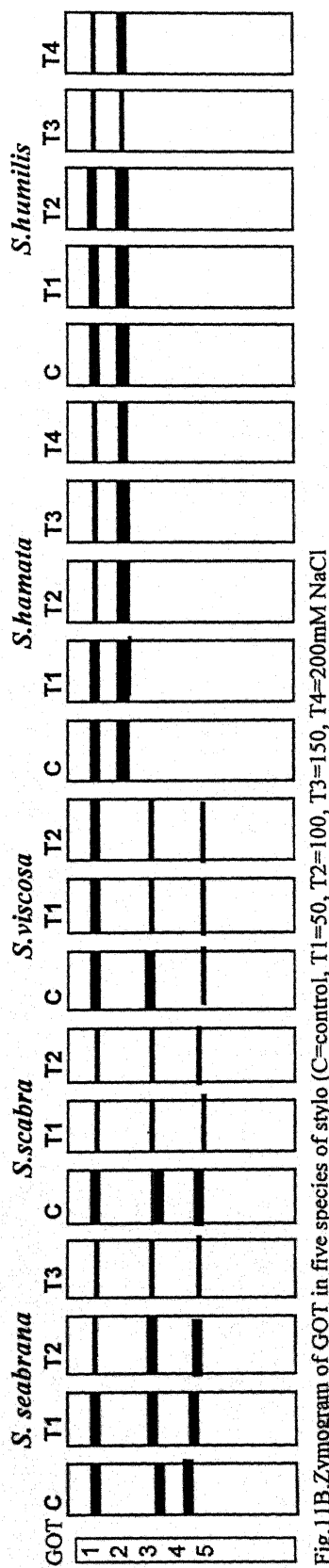
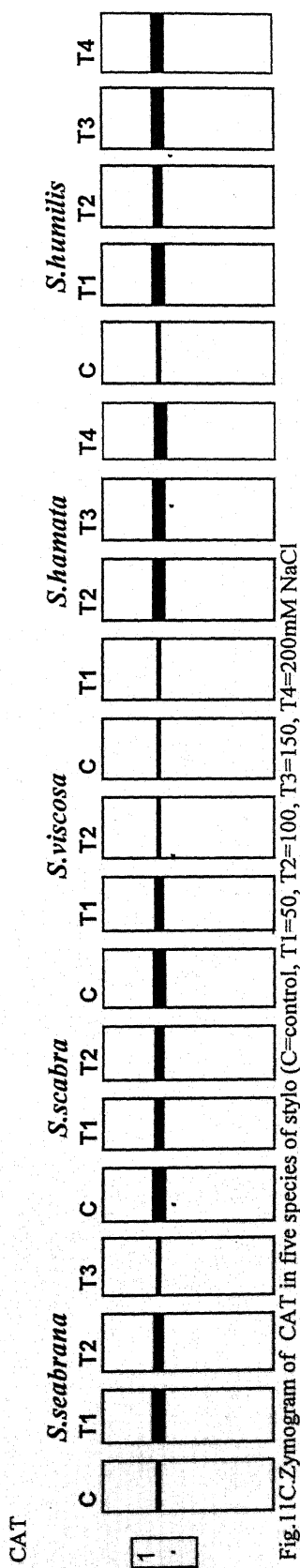


Fig 11C: Isozyme patterns of catalase, peroxidase, poly phenol oxidase in five different species of stylo under control (C), 50mM (T1), 100 mM (T2), 150 mM (T3)



during stress in both *S. viscosa* and *S. hamata*. PRX-1 disappeared at 100mM of stress and again reappeared at 150mM of stress. In *S. humilis* PRX-1 and PRX-4 disappeared at 100mM stress, while PRX-1 reappeared at 150mM. PRX-3 isoform remains unchanged. Intensity of PRX-2 isoform decreased at 100mM stress and then increased at 150mM of stress (Fig. 11C and 11L)..

#### **Acid phosphatase (ACP)**

In total 10 isoforms of ACP was visualized. In *S. seabrana* intensity of ACP-10 isoform was increased at 50mM of stress, whereas ACP-1, 2, 6, 9 and 10 isoforms remains unchanged. In *S. scabra* ACP-4, 5 and 9 showed increase in intensity at 100mM stress and three isoforms namely ACP-1, 2, 3 and 7 showed no change over control. In *S. viscosa* out of seven isoforms, five namely ACP-1, 2, 3, 4 and 7 isoforms remains unchanged under stress whereas isoforms ACP-5 and 9 increased in stress. In *S. hamata*, intensity of ACP-2 isoform increased at 150mM stress while ACP-4 disappeared at 100mM stress level. Decrease in intensity at 200mM stress level was observed of isoforms ACP-7. Six isoforms namely ACP-1, 3, 5, 8 and 10 showed no change. Four isoforms of *S. humilis* namely ACP-1, 3, 7, and 10 did not show any change. ACP-2 increased at stress level of 50mM but decreased at 100mM stress. ACP-4 showed increase in intensity at 50mM stress level but it disappeared at 150mM of stress level. Four isoforms namely ACP-1, 3, 7 and 10 showed no change in stress over control (Fig. 11A and 11H).

#### **Malate dehydrogenase (MDH)**

Out of five isoforms observed in *S. seabrana* (MDH-1, 2, 3, 4 and 5), intensity of MDH-2 increased at 100mM stress level. Two isoforms namely MDH-4 and 5 in *S. scabra* increased at 50mM stress level whereas MDH-5 decreased at 100mM. Two isoforms MDH-1 and 3 remains unchanged upon stress imposition. In *S. viscosa* intensity of MDH-4 increased whereas MDH-3 showed no change over stress. In *S. hamata*, four isoforms namely MDH-1, 2, 3, and 4 showed no change over stress but MDH-5 disappeared at 100mM stress which reappeared at 150mM of salt stress. In *S. humilis* isoforms MDH-1 and 2 appeared only at stress level of 150mM and MDH-5 appeared

from the very low level of stress i.e., 50mM. MDH-4 increased at 50mM and 150mM whereas MDH-3 showed no change over stress condition (Fig. 11A and 11I).

#### **Isocitrate dehydrogenase (IDH)**

Single isoform of IDH was observed in all species of *Stylosanthes*. Only at stress level of 150mM IDH isoform showed quantitative change in *S. seabrana*. However, *S. scabra*, *S. hamata* and *S. humilis* showed increase in the intensity at 100mM stress level. In *S. viscosa*, isoform remains unchanged over stress (Fig. 11E and 11Q).

#### **Leucine aminopeptidase (LAP)**

As IDH isozyme LAP also showed single isoform in all species of *Stylosanthes* even in stress condition. Increased intensity was observed at 50mM of stress which decreased and after 150mM of stress level it increased. In *S. scabra* increase in intensity was observed which decreased at 100mM of stress. LAP isoform increased in *S. viscosa* at 50mM of stress level. In *S. hamata* and *S. humilis* increased change was observed at 50mM of stress which remains unchanged during higher stress levels (Fig. 11E and 11R).

#### **Glutamate dehydrogenase (GDH)**

In total four isoforms namely GDH-1, -2, -3 and 4 was observed in *Stylosanthes*. In *S. seabrana* GDH-1, 2 and 4 and in *S. scabra* GDH-1 and 4 showed no change upon salt imposition. Whereas in *S. viscosa* intensity of GDH-4 isoform was increased at 100mM stress. No change in isoforms GDH-1 and 5 was observed in *S. viscosa* in various stress stages, while isoforms GDH-4 decreased at 150mM and then increased at 200mM stress. In *S. humilis* isoform GDH-3 was newly formed at the stress level of 150mM while GDH-1 and 5 showed no change in stress (Fig. 11M).

#### **Phosphoglucomutase (PGM)**

In total five isoforms of PGM namely PGM-1, -2, -3, -4 and 5 was observed in *Stylosanthes*. Two new isoforms namely PGM-4 and 5 was observed in *S. seabrana* at 150mM of stress level. PGM-1 decreased at 50mM at then increased at 100mM and further decreased at 150mM of stress level. In *S. scabra* PGM-1 decreased after salt

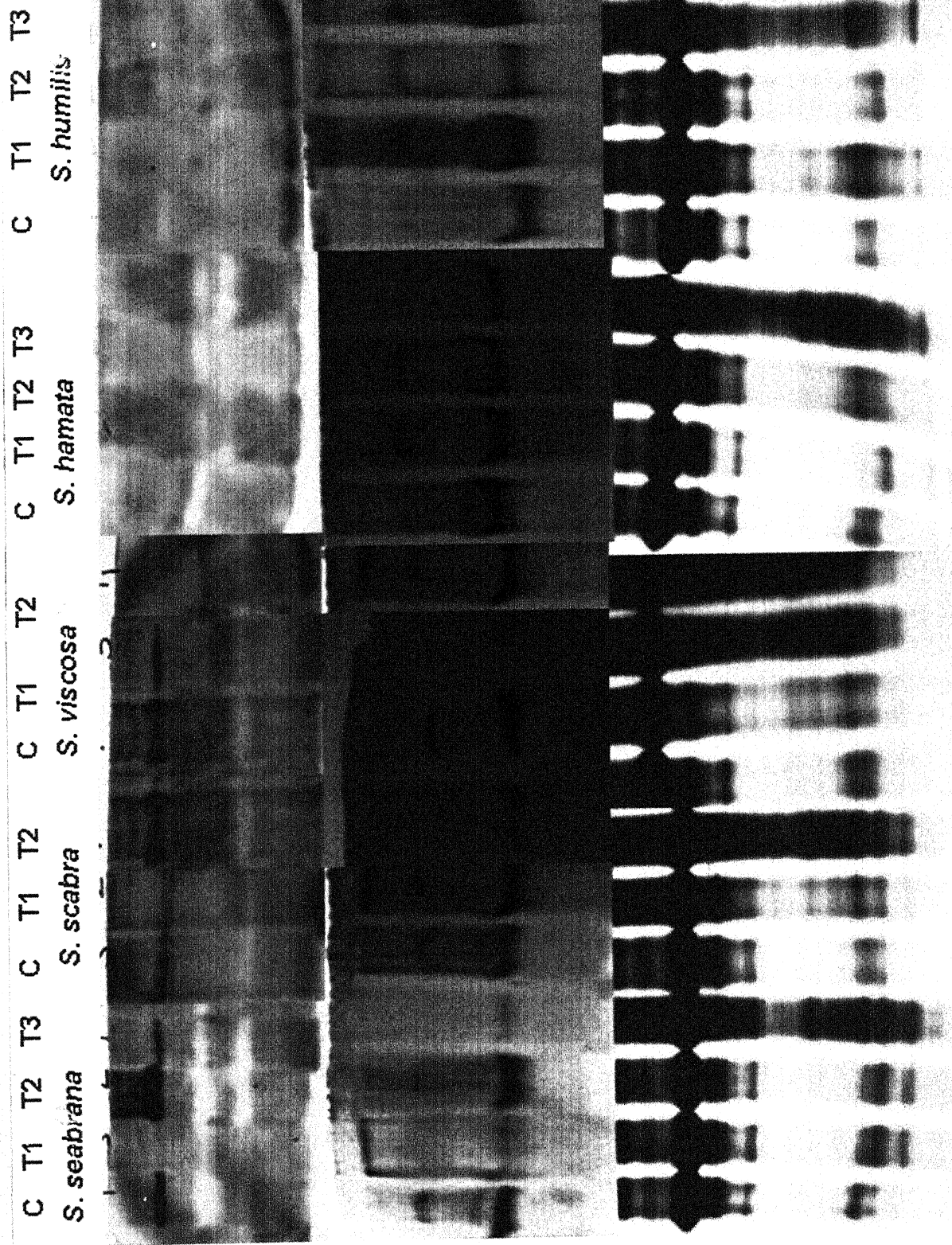


Fig 11D: Isozyme patterns of phospho glucomutase, fructose biphosphate aldolase and native protein profile in five different species of stylo under control (C), 50mM (T1), 100 mM (T2), 150 mM (T3)

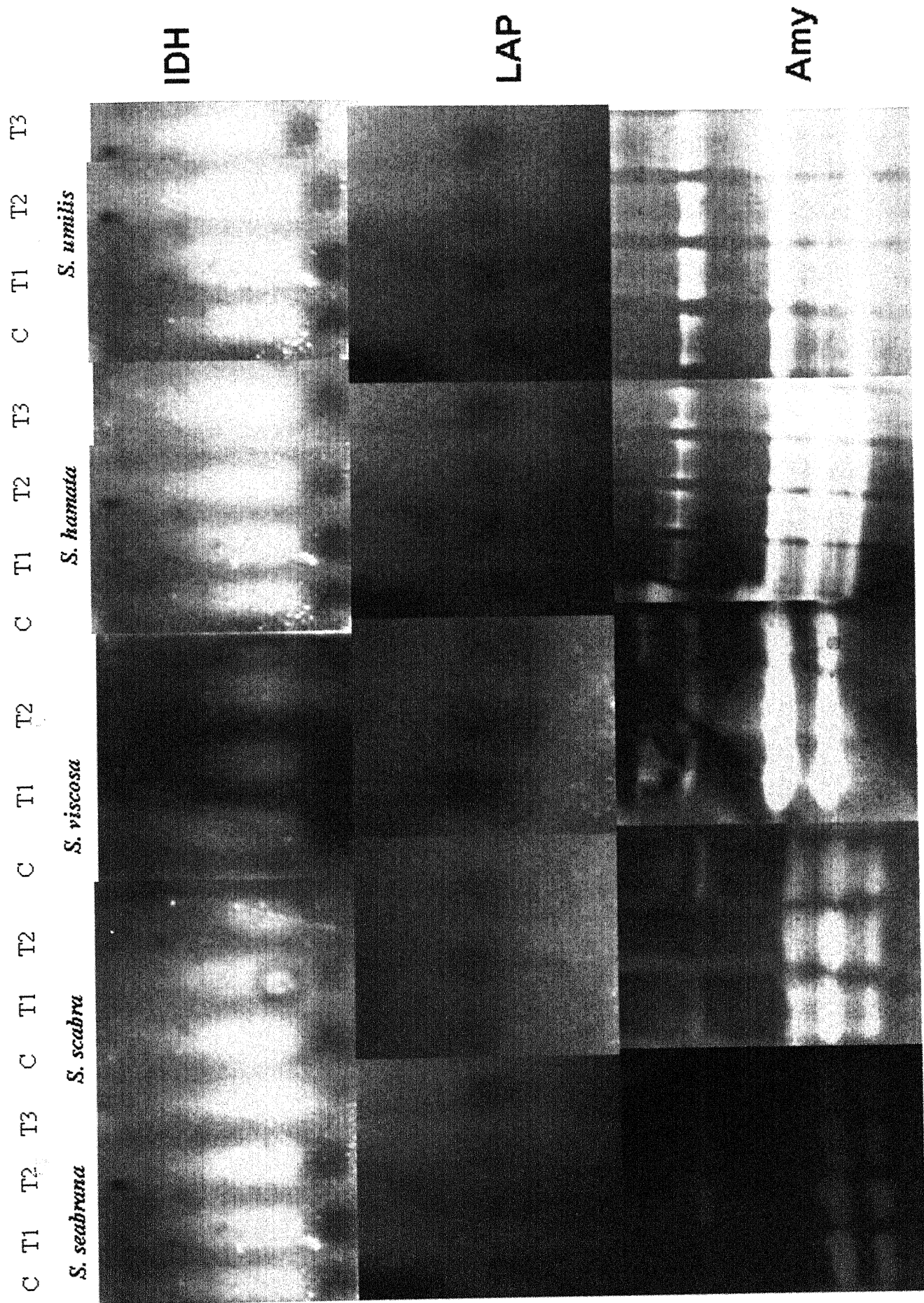


Fig. 11E: Isozyme patterns of isocitrate dehydrogenase, leucine amino peptidase, amylase in five different species of stylo under control (C), 50mM (T1), 100 mM (T2), 150 mM (T3)

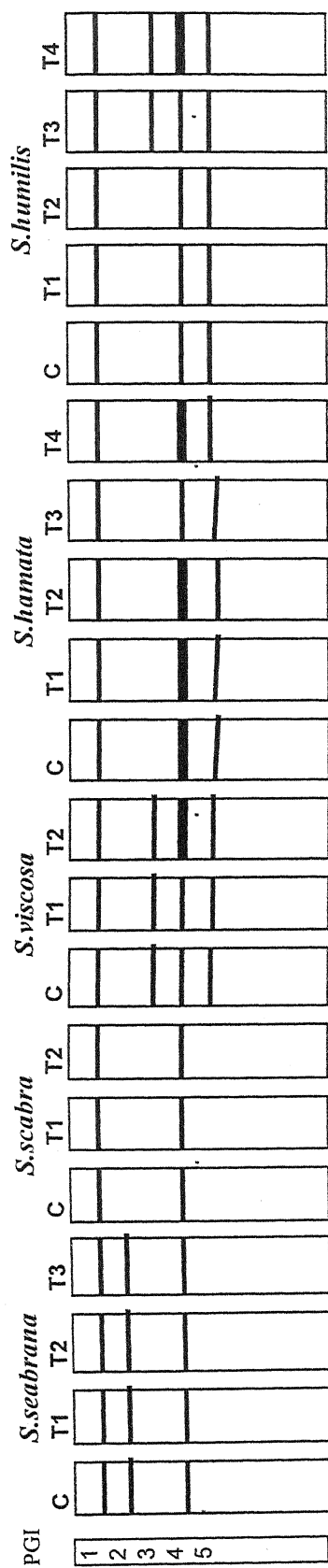


Fig. 11A. Zymogram of PGI in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

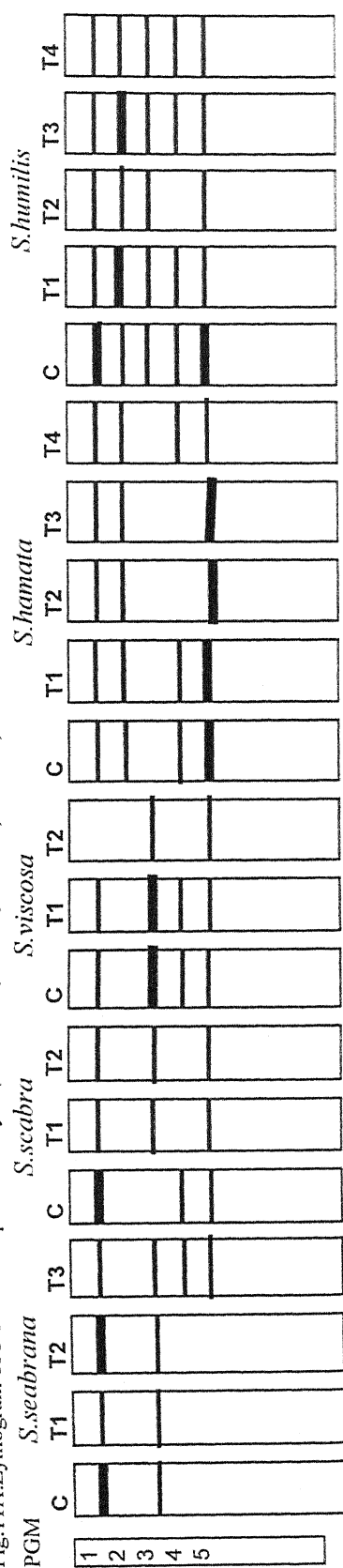


Fig. 11B. Zymogram of PGM in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

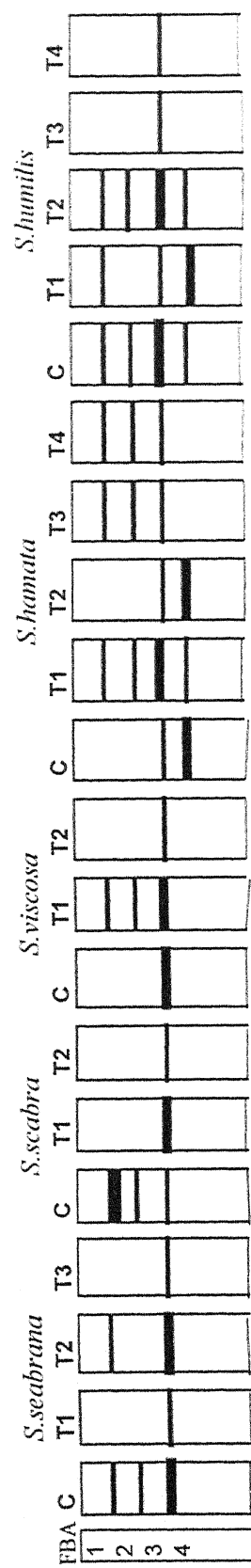


Fig. 11D. Zymogram of FBA in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

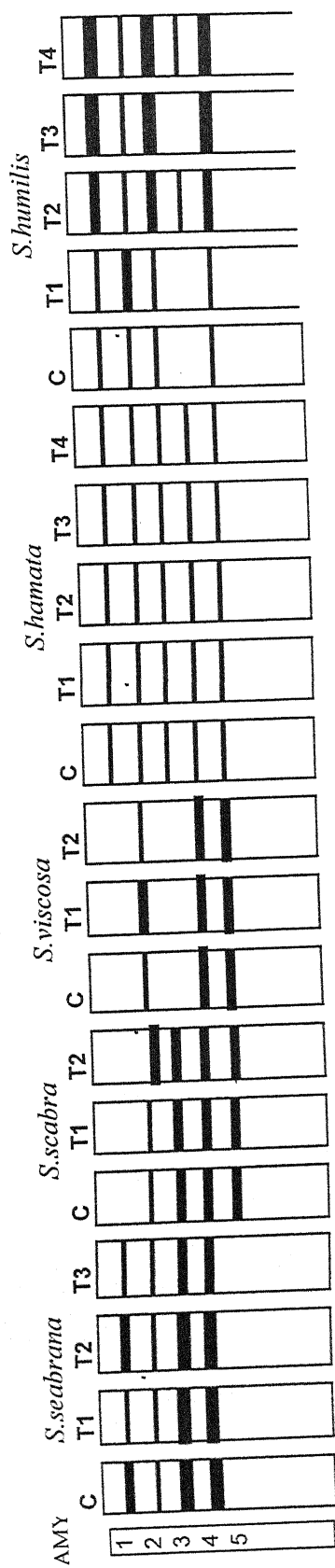


Fig.11E.Zymogram of AMY in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl

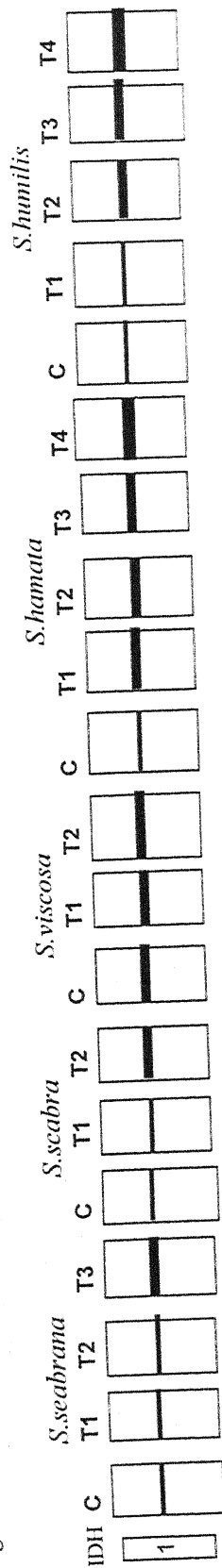


Fig.11E.Zymogram of IDH in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl

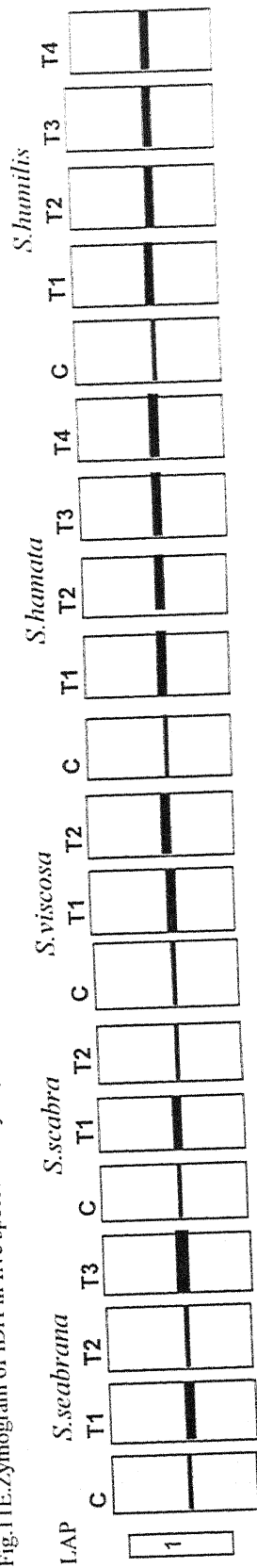
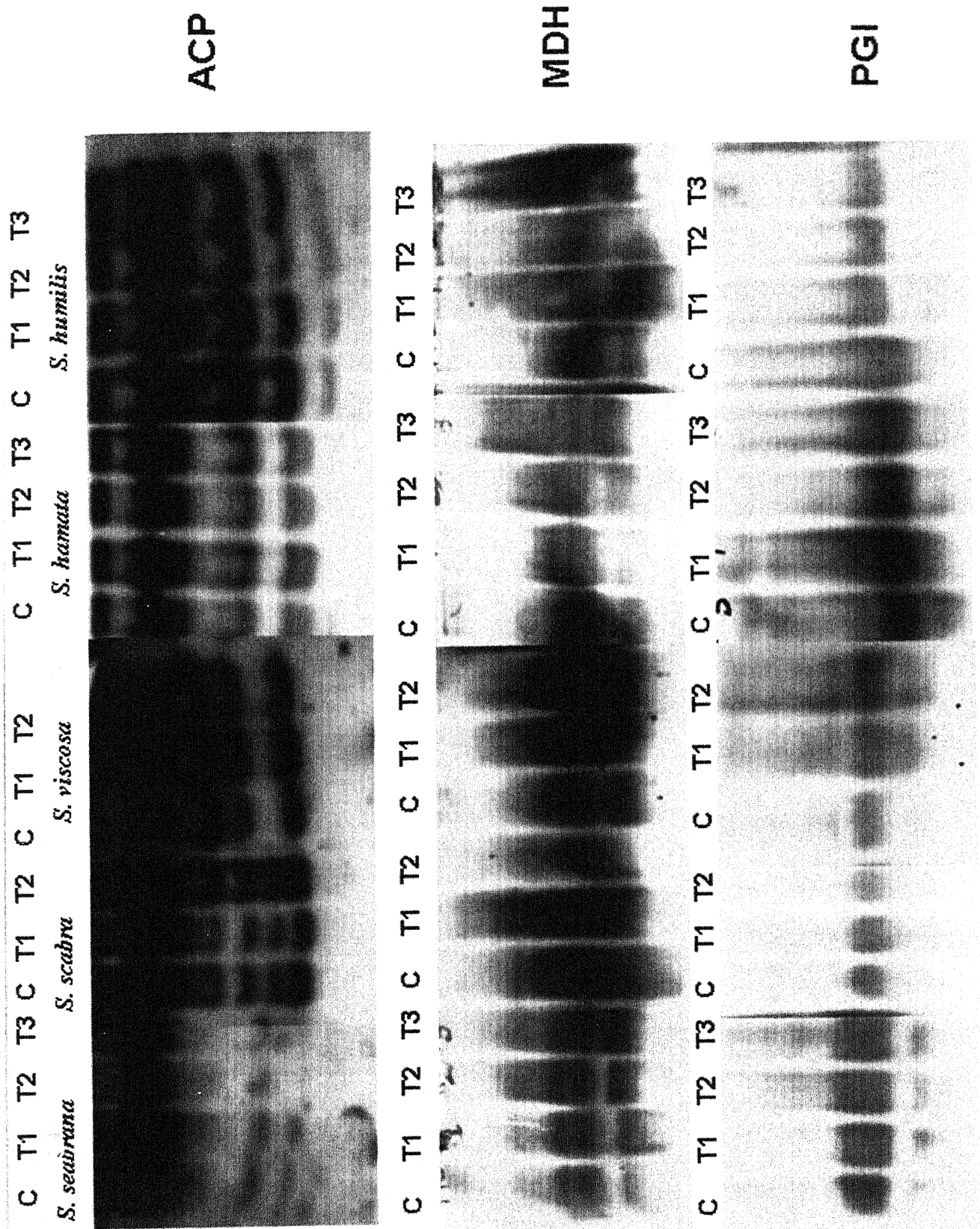


Fig.11E.Zymogram of LAP in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl



**Fig 11A: Isozyme patterns of Acid phosphatase, malate dehydrogenase, phosphoglucose isomerase in five different species of stylo under control (C), 50mM (T1), 100 mM (T2), 150 mM (T3)**

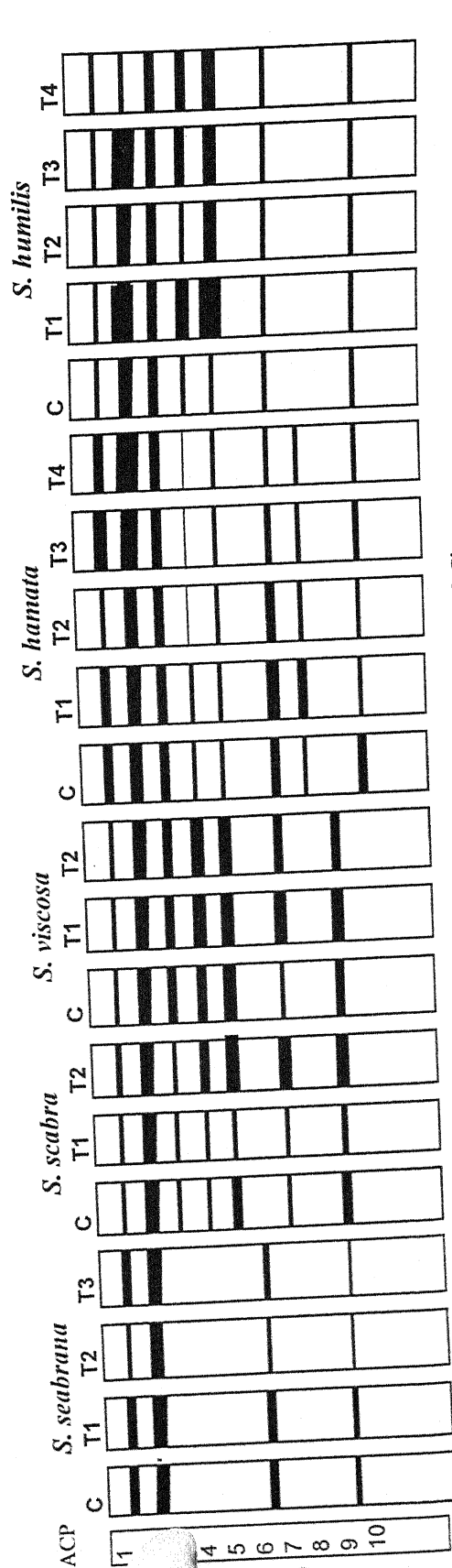


Fig.11A. Zymogram of ACP in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

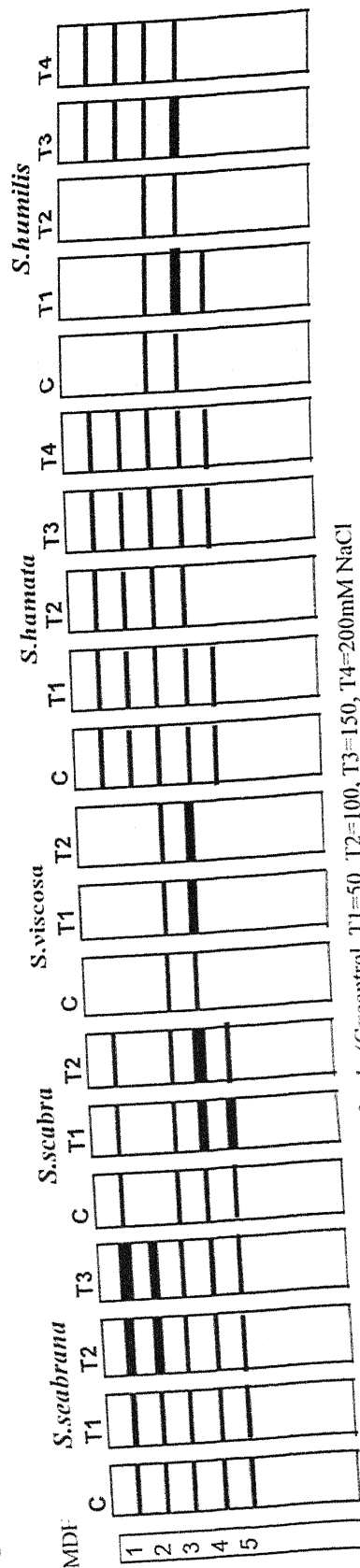


Fig.11A. Zymogram of MDH in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

imposition. PGM-3 was newly formed at 50mM of stress while PGM-4 appeared only in control condition. PGM-5 showed no change in both *S. scabra* and *S. viscosa* even under stress. In *S. viscosa*, two isoforms namely PGM-1 and 4 disappeared at 100mM of salt stress. Decreased intensity of isoform PGM-3 was observed at 100mM of stress. In *S. hamata* two isoforms namely PGM-1 and 2 exhibited no change over stress while PGM-4 formed as a new isoform and PGM-5 showed decrease in intensity at 200mM of stress. Intensity of isoform PGM-4 and 5 in *S. humilis* decreased from 50mM of stress level. PGM-1 and 3 showed no change in stress conditions. PGM-4 disappeared at 100mM of stress and again it reappeared at stress level of 150mM (Fig. 11D and 11N).

#### **Fructose biose aldolase (FBA)**

In total four isoforms namely FBA-1, -2, -3 and 4 was observed in *Stylosanthes*. Isoforms FBA-1 and FBA-2 in *S. seabrana* disappeared at 50mM stress while FBA-1 reappeared at 100mM and again disappeared at 150mM of stress. FBA-4 decreased at 50mM of stress and then increased at 100mM. In *S. scabra*, isoforms FBA-1 and 2 disappeared on exposure to salt stress. FBA-4 increased at 50mM and then decreased at 100mM of stress. Two new isoforms FBA-1 and 2 appeared only at 50mM of stress and isoform FBA-3 decreased at 100mM in both *S. viscosa* and *S. hamata*. In *S. hamata* isoform FBA-1 and 2 first appeared at 50mM and then disappeared at 100mM of stress and again reappeared at 150mM of stress. Intensity of isoform FBA-3 was increased at 50mM of stress level. FBA-4 in *S. hamata* showed decrease in intensity at stress level of 50mM and at 100mM it again increased and further disappeared at higher stress. FBA-1 did not show any increase in intensity while it disappeared at 150mM of stress level. FBA-2 of *S. humilis* disappeared at start of salt stress and then it reappeared at 100mM of stress and at 150mM it again disappeared. FBA-3 decreased at 50mM and then increased at 100mM of stress and at higher level of stress it remains as a control. FBA-4 increased at 50mM of stress level and it disappeared at 150mM of stress (Fig. 11D and 11O).

#### **Amylase (AMY)**

In total five isoforms was observed in *Stylosanthes*. Intensity of three isoforms AMY-1, 3 and 4 decreased in *S. seabrana* at stress level of 150mM. AMY-2 remains unchanged

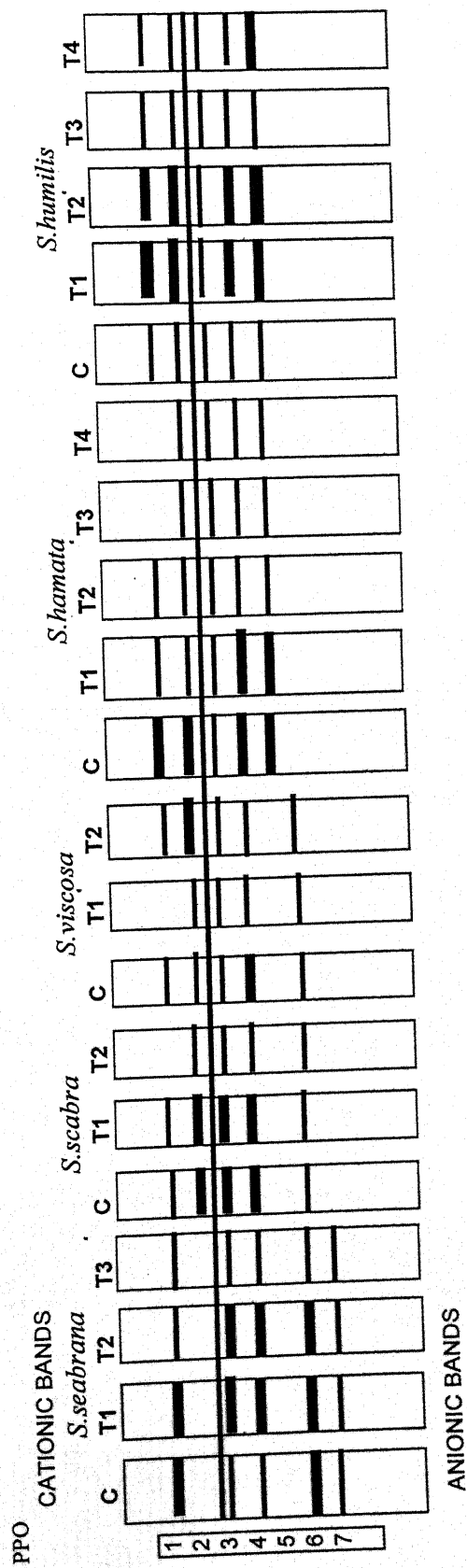


Fig. 11C. Zymogram of PPO in five species of stylo (C=control, T1=50, T2=100, T3=150, T4=200mM NaCl)

over salt stress. In *S. scabra* three isoforms namely AMY-3, 4 and 5 showed no change in stress stages while AMY-2 increased at 100mM of salt stress. In *S. viscosa*, AMY-4 and 5 showed no change but AMY-2 increased at stress level of 50mM. In *S. hamata*, no change was observed. AMY-1, 3 and 5 increased in their intensity at stress level of 100mM. AMY-2 showed decrease in intensity from the stress level of 100mM. A new isoform, AMY-4 appeared in only 100mM and 200mM of stress in *S. hamata* (Fig. 11E and 11P).

### **Polyphenol oxidase (PPO)**

In total seven isoforms of PPO (PPO-1, 2, 3, 4, 5, 6 and 7) was observed in *Stylosanthes*. The cationic isoform PPO-1 decreased at stress level of 100mM, while anionic PPO-3 and PPO-4 showed increase in intensity over control. Two isoforms namely PPO-6 and 7 exhibited no change over control. PPO-1 disappeared at stress level of 100mM while PPO -2, -3 and 4 showed decrease in activity at stress level of 100mM. PPO-6 showed no quantitative change over stress in *S. scabra* and *S. viscosa*. In *S. viscosa*, isoform PPO-1 disappeared at 50mM of stress and reappeared at 100mM. Increase in intensity was observed in PPO-2 and PPO-4 at the level of 50mM. PPO-1, 2, 4 and 5 decreased in 50mM of stress. PPO-3 showed no change both in *S. hamata* and in *S. humilis*. Intensity of PPO-1, 2, 4 and 5 increased at 50mM of stress and again decreased at the level of 150mM (Fig. 11C and 11S).

### **Native protein gel electrophoresis:**

In total 14 bands were observed in 12% native gel. Intensity of band-1 was increased in all species of *Stylosanthes* during salt stress. In *S. seabrana* bands-2, 3, 4, 5 and 6 showed no qualitative as well as quantitative change. Intensity of band-7 increased at 50mM and decreased at 100mM and then again increased at 150mM. Intensity of bands-8, 9 increased in 150mM of stress level. Band-10 intensity increased in control as well as in 150mM of stress to that of other level of stress. Intensity of band-11 and 13 increased at 50mM and was maximum at 150mM. Band-12 was appeared only in 150mM of stress level. In *S. scabra* intensity of bands-2, 3, 4, 5, and 6 were same, whereas band-7, 8, 9,

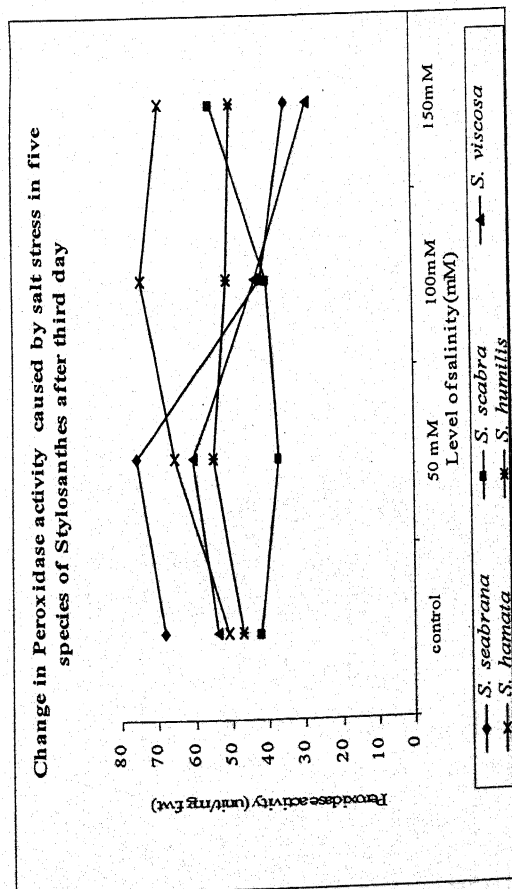
10, 11 and 13 showed quantitative increase. *S. viscosa* showed no change in bands-2, 3, 4, 5, and 6. However intensity of bands-7 decreased in 50mM of stress. Bands-8, 9, 10, 11 and 13 showed quantitative increase in stress stages. In *S. hamata* at the stress level of 200mM decrease in intensity of all bands was observed. Bands-2 and 3 showed no change while band-4, decreased at 50mM and then increased at 100mM of stress level. Intensity of band-6 increased at 50mM and then decreased at the stress level of 100mM. Band-7 decreased at 50mM and showed increase in intensity at 100mM of stress. Band-8 and 9 increased up to the stress level of 150mM. Band-10 decreased at 50mM of stress and again it increased at 150mM of stress. Band-11 disappeared at 50mM of stress level and it reappeared at stress level of 100mM. Band-13 was observed only in stress of 150mM. Band-2 and 3 showed no change in *S. humilis* while band-4 increased and further decreased at 100mM of stress. Band-5 showed decrease in intensity. Band-6 and 7 increased at 50mM of stress and further decreased at stress level of 100mM, further increased at 150mM. At 50mM and 150mM band-8 and 9, 10 increased and then decreased at 100mM of stress. Band-11 increased at 50mM of stress and then decreased at 100mM. Quantitative increase was observed in band-12 up to 150mM of stress (Fig. 11D and 11T).

#### Enzyme activities in seedlings:

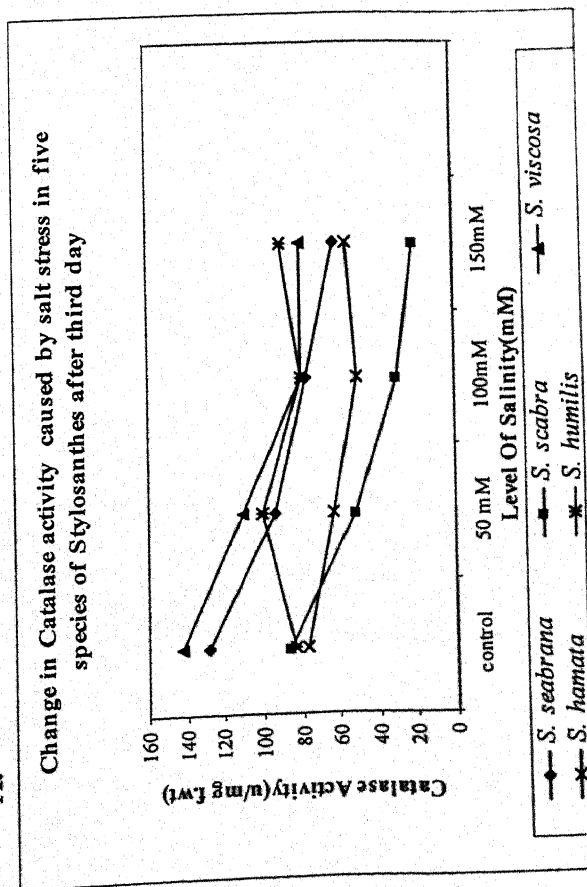
Seedlings of *Stylosanthes* was germinated on petri plate with moist filter paper. Apart from control stress of 50mM, 100mm, 150mM and 200mM NaCl was given. Enzyme activity data was recorded on third and fifth day of stress.

#### Peroxidase (PRX)

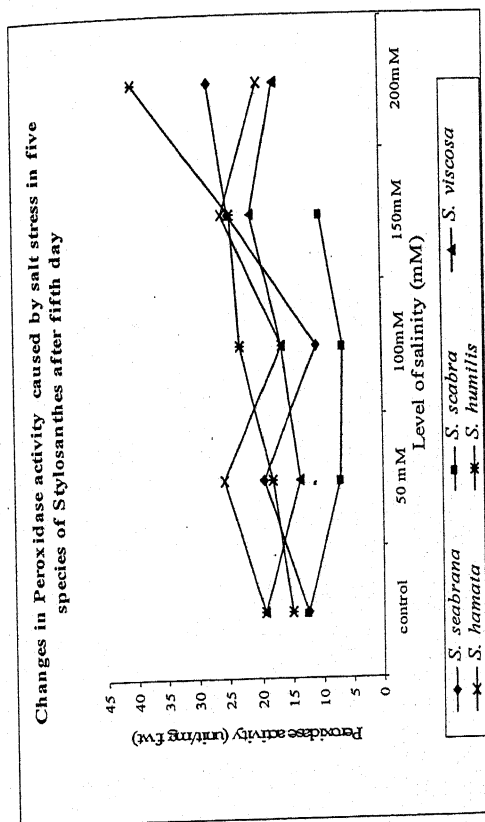
In general when seedlings was germinated in presence of NaCl increase in peroxidase activity was observed in *S. hamata* and *S. humilis* and decrease in *S. viscosa*, *S. seabrana* and *S. scabra* species (Fig. 12A). At lower level of salinity (50mM) increase in activity was observed in all five species, when activity was measured after three days of stress. The increase in activity was minimum *S. humilis* (46.8 to 49.3 unit/mg fr. wt) (Table 2). In case of *S. hamata* activity increased from 50.8 to 68.6 unit/mg fr. wt. Even after five



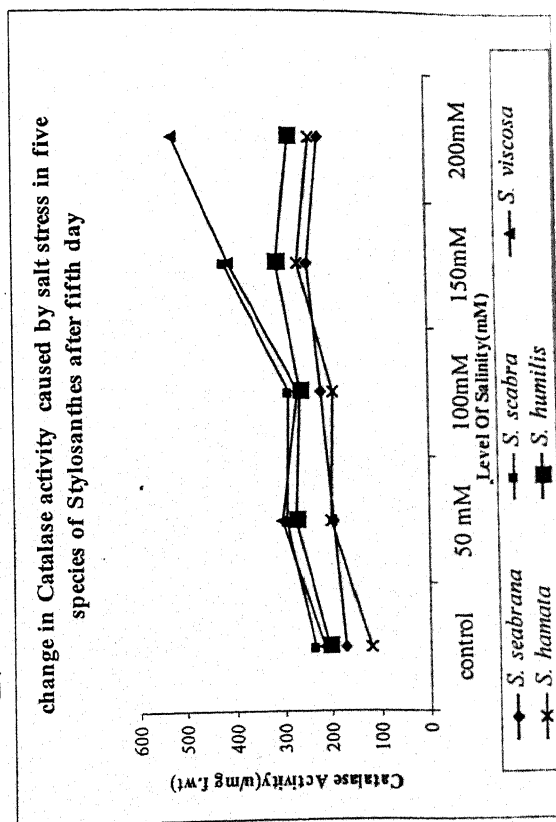
A.



C.



B.



D.

Fig 12A: Change in Peroxidase (top) and Catalase (bottom) activity. A and C = Third day, B and D = Fifth day in seedlings at different levels of salinity.

**Table 2.** Activities (unit/gm fr. wt. min) of Peroxidase (A), Catalase (B) and Superoxide dismutase (C) in five species of stylo measured at 3rd and 5th day of salinity stress.

(A)

Species	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	68.3	75.53	41.6	34.56	1055	775	998.3	796.67
<i>S. scabra</i>	42	36.8	40	55	188.3	515	161.6	490
<i>S. viscosa</i>	53.8	60.4	43.06	28.45	348.3	175	225	823.33
<i>S. hamata</i>	50.8	64.95	74.2	68.6	756	988.3	506.67	1676.67
<i>S. humilis</i>	46.8	54.8	50.8	49.33	666.67	1091.67	660	981.67

(B)

	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	127.86	92.93	77.33	61.96	174	194.5	221.5	250
<i>S. scabra</i>	85.26	51.7	29.66	20	235	294	290	422
<i>S. viscosa</i>	142.46	110.4	79.43	79.8	215	307	270.5	412
<i>S. hamata</i>	76.93	62.8	50	55.46	116	202	198	272
<i>S. humilis</i>	83.33	100.33	79.066	89.86	206	274	264	316

(C)

	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	168.070	176.630	156.300	125.500	134.25	116.25	120	90.3
<i>S. scabra</i>	85.83	115.9	91.37	93.57	117	111	92.7	88.2
<i>S. viscosa</i>	98.63	121.87	97.83	86.3	99.7	95.95	91	129
<i>S. hamata</i>	149.63	164.93	127	110.3	113.5	136.95	115.05	100.5
<i>S. humilis</i>	71.33	98.60	71.93	58.67	165.35	127.15	114.9	82.5

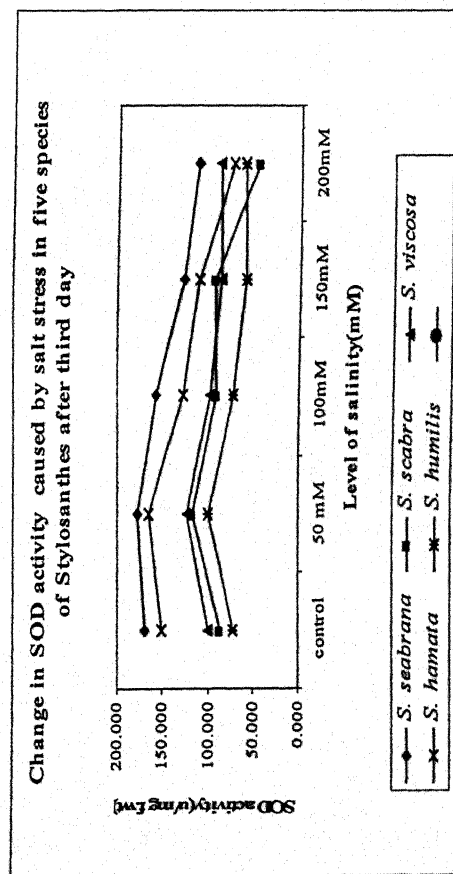
days of stress, *S. hamata* and *S. humilis* showed increase in activity in comparison to control (Fig. 12 A). At higher level of salinity i.e., 200mM significant increase in activity was observed only in *S. humilis* (15.04 to 40.0 unit/mg fr. Wt). At higher level of salinity i.e. more than 150mM, *S. scabra* also showed increase in PRX activity. At 200mM level of NaCl, *S. scabra* did not survive after five days of stress hence activity was not measured.

### Catalase (CAT)

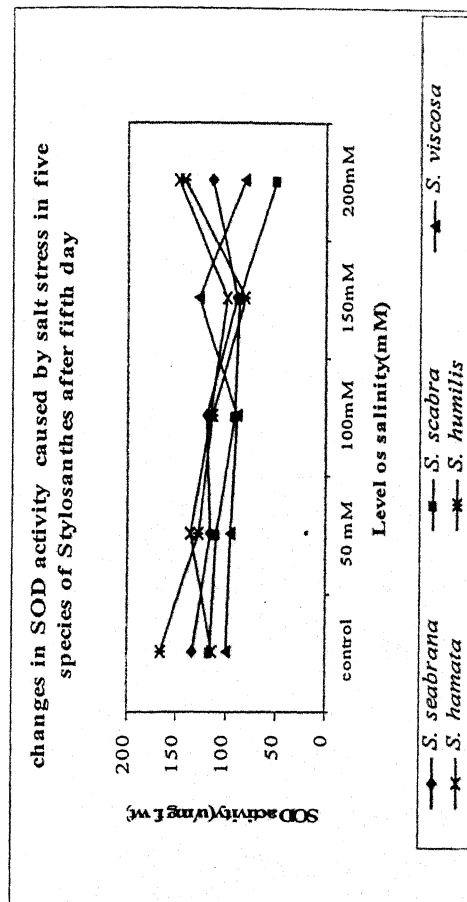
In general decrease in activity was observed in five species of *Stylosanthes* measured at four levels 50, 100, 150 and 200mM (Fig. 12A). Maximum decrease in activity was observed in *S. seabrana* (127.86 to 61.96 unit/mg fr. wt) whereas minimum was in *S. hamata* (76.93 to 55.46 unit/mg fr. wt) (Table 2). A slight increase in activity was observed in *S. humilis* at 100mM of NaCl stress. At 150mM decrease in activity was observed. Minor increase in activity at 200mM was observed in all five species. When activity was measured at fifth day of stress increase in CAT activity at 50mM of NaCl level was observed. At 100mM stress level all species showed decrease in CAT activity. In general increase in CAT activity was observed after five days of NaCl stress. Maximum increase was found in *S. viscosa* at 200mM concentration of NaCl (529.5unit/mg fr. wt). After longer exposure of seedlings at higher level of salinity the increase in catalase activity was observed in all five species.

### Superoxide dismutase (SOD)

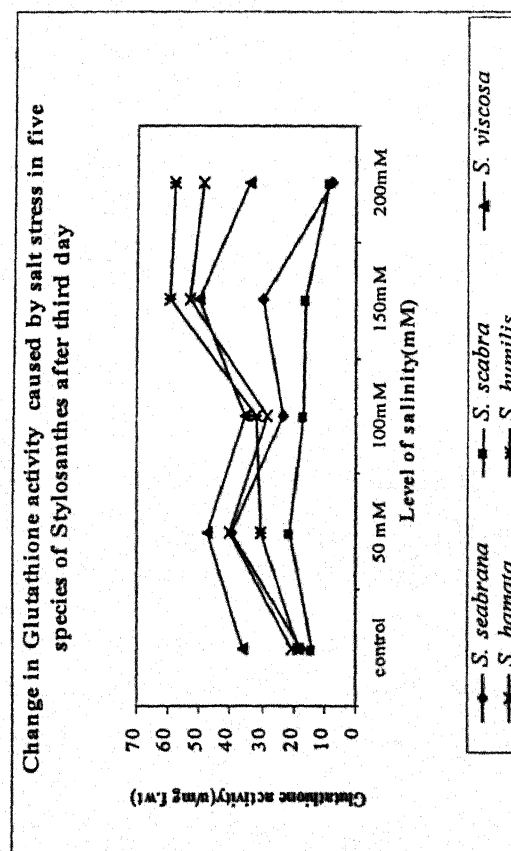
Superoxide dismutase enzyme activity was measured in five species of *stylosanthes* under control and salt stress condition and represented as units/ mg fr. wt. min<sup>-1</sup>. At low level of stress increase in activity was observed which further decreased after increase in the level of salinity stress (Table 2). After three days of stress maximum decrease was observed in *S. hamata*, whereas minimum in *S. humilis* (Fig. 12B). When duration of stress was increased i.e., from three days to five days, SOD activity was observed increase in all five species. At lower level of salinity stress SOD activity was increased at third day whereas at higher level of stress increased activity declined in due course of time. At fifth day of stress, SOD activity decreased linearly with the increasing stress



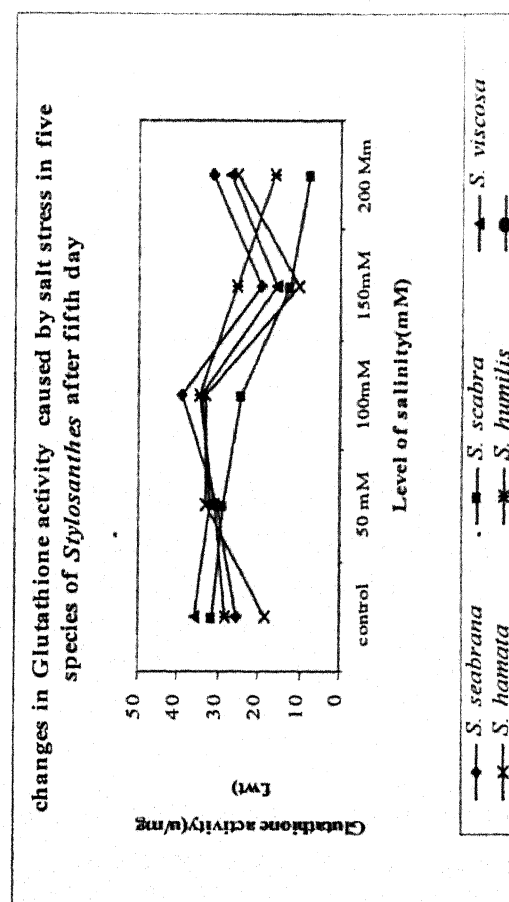
A.



B.



C.



D.

Fig 12B: Change in SOD (top) and Glutathione reductase (bottom) activity A and C = Third day B and D = Fifth day in seedlings at different levels of salinity.

level. *S. scabra* specifically showed decrease in activity on 3<sup>rd</sup> and 5<sup>th</sup> day of stress (Fig. 12 B).

#### Glutathione reductase (GR)

At 50mM stress level of NaCl GR activity increased whereas at 100mM rapid decrease in activity was observed which further increased when stress level was increased to 150mM (Fig. 12B). At 200mM of stress level decreased activity was observed. In general *S. seabrana*, *S. scabra* and *S. viscosa* showed decrease GR activity at third day (Table 3). Increased activity after third day of NaCl stress was found in *S. hamata* and *S. humilis* (Fig. 12 B). At fifth day of stress increase in activity of glutathione reductase was observed at low level of stress whereas at higher level of stress the activity decreased.

#### Glycolate oxidase (GO)

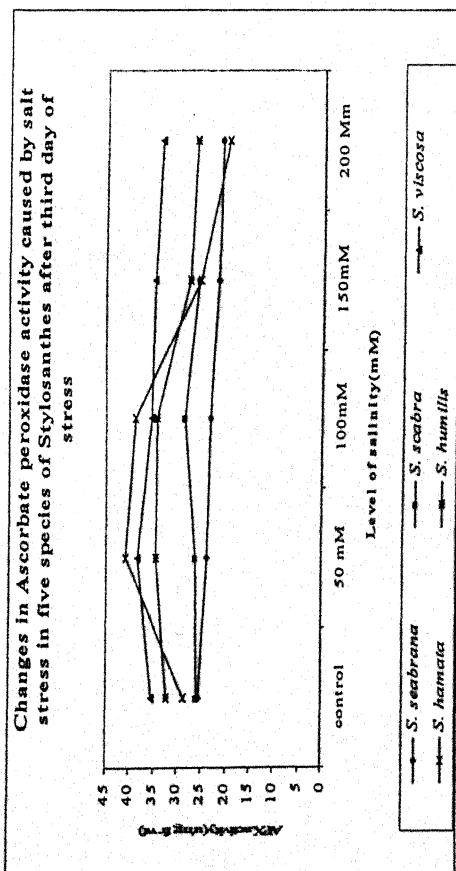
Glycolate oxidase activity measured at different days of stress showed minor increase in activity at 50mM stress, except *S. seabrana*, whereas at 100mM, *S. hamata* showed no change in activity. An increase in activity, at higher level of stress was observed on third day of stress. Activity of glycolate oxidase increased linearly with the increase in the level of stress. Maximum increase in activity at 200mM NaCl stress was observed in *S. hamata* (31.96 to 94.12 unit/mg fr. wt) (Table 3). Increased activity of enzyme in *S. hamata* and *S. humilis* was observed on fifth day of stress, whereas *S. seabrana*, *S. scabra* and *S. viscosa* showed decreased glycolate oxidase activity. At lower level of stress activity remains constant as in control but at 200mM of NaCl stress increased activity was observed (Fig. 12 C).

#### Ascorbate peroxidase (APX)

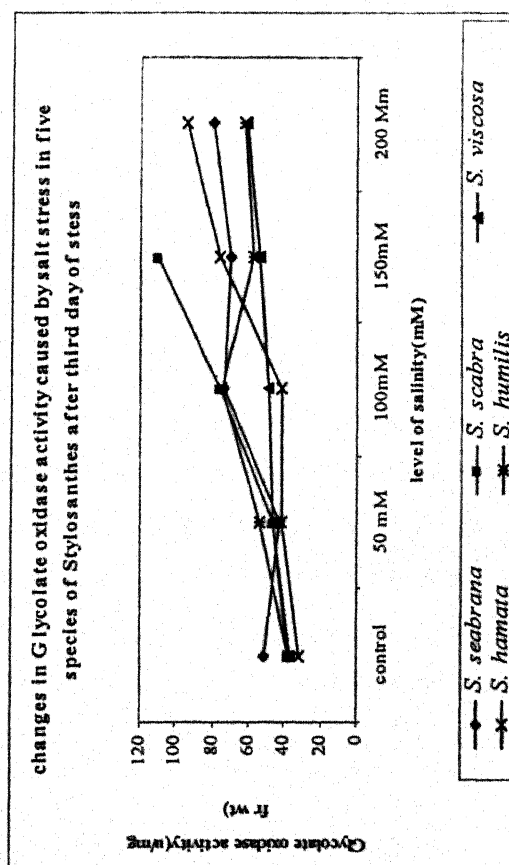
Ascorbate peroxidase activity measured at different levels of stress indicated significant change in five species of *Stylosanthes* at third day of stress. Increased APX activity at 50mM, 100mM and 150mM stress level was observed in *S. hamata*. At 200mM of NaCl stress decreased activity was observed in all species (Table 3). At fifth day of stress, however at low stress i.e., at 50mM, increased activity was found where as at higher stress, activity decreased. Except *S. viscosa* which showed minimum decrease (79.2-58.0

**Table 3.** Activities (unit/gm fr. wt./min) of Glutathione reductase, Glycolate oxidase and Ascorbate peroxidase in five species of stylo measured at 3rd and 5th day of salinity stress.

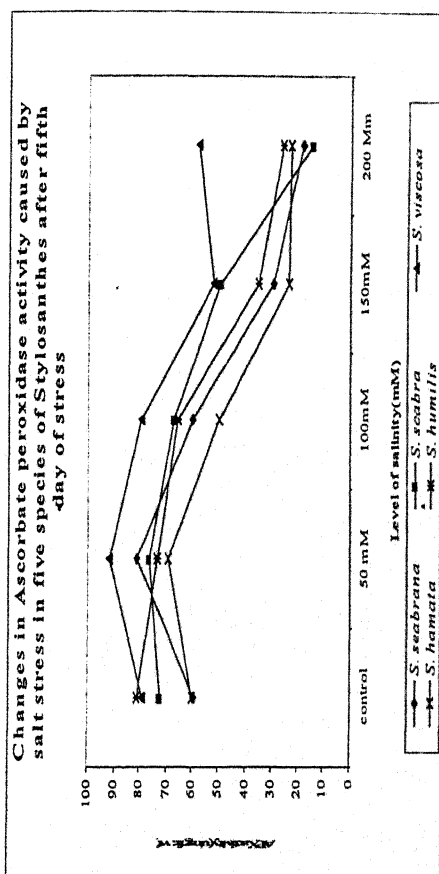
	Glutathione reductase (U/gm fr. wt./min)							
	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	17.9	39.5	23.1	29.5	25.5	30.2	39	19.5
<i>S. scabra</i>	14	21.5	17.1	16.1	31.65	28.9	24.6	12.75
<i>S. viscosa</i>	36.2	47.3	35.6	50	36.05	32.3	34.35	15.55
<i>S. hamata</i>	20.6	40.4	28.2	53.2	18.5	33.3	33	10.5
<i>S. humilis</i>	18.3	30.3	31.7	59.4	28.35	31.05	35.05	25.5
	Glycolate oxidase (OD/gm fr. wt./min)							
	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	50.838	43.068	73.926	70.152	40.959	52.281	49.284	47.619
<i>S. scabra</i>	38.406	46.842	75.924	110.778	52.281	51.28	46.287	28.638
<i>S. viscosa</i>	37.296	46.176	49.284	53.502	42.957	44.622	35.298	44.289
<i>S. hamata</i>	31.968	41.736	41.07	76.368	44.955	47.286	42.291	45.288
<i>S. humilis</i>	38.406	54.39	75.258	57.74	49.617	52.947	51.948	57.276
	Ascorbate Peroxidase (U/gm Fr. wt./min)							
	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	25.5	23.75	23.125	21.625	59.5	81	59.95	29.75
<i>S. scabra</i>	26.125	26.25	28.625	25.625	72.5	76.5	67.6	49.35
<i>S. viscosa</i>	35.375	38.375	35.5	34.75	79.2	91.75	79.35	51.9
<i>S. hamata</i>	28.75	40.75	38.875	25.375	60.35	69.3	50	23.5
<i>S. humilis</i>	32.125	34.5	34.125	27.5	81	73.25	66	35.25



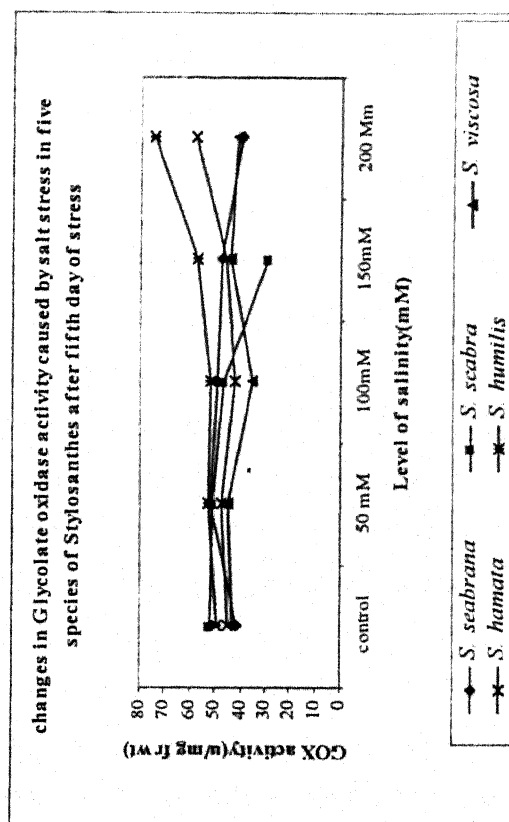
**A.**



**C.**



**B.**



**D.**

Fig 12C Change in Ascorbate peroxidase (top) and Glycolate oxidase (bottom) activity A.C. Third Day B.D. Fifth Day in seedlings at different levels of salinity

u/mg f.wt), all other species showed major decrease in ascorbate peroxidase activity (Fig. 12C).

#### **Protein content:**

Total soluble protein content was measured in five species of *stylosanthes* under control and salt stress condition. Total soluble proteins measured at different days of salt stress indicated increase in its content with increasing magnitude of stress. In control the level of soluble proteins in five species of *Stylosanthes* ranged from 22.296 to 33.744 mg/g f.wt (Table 4A). At 50mM stress level, major increased in protein content was observed in *S. seabrana*, *S. hamata* and *S. humilis* whereas *S. scabra* and *S. viscosa* showed minor increase. No change in protein content in *S. scabra* was observed at 100mM NaCl stress. Decreased protein content was observed at 150mM in all species of *Stylosanthes*. Minor decrease in protein level was shown by all species at 200mM stress. After three days of stress, protein level increased significantly in all five species of *Stylosanthes*. Maximum increase in protein content was observed in *S. hamata* (16.114 mg/gm fr. wt), whereas minimum in *S. scabra*. In general increase in protein content was observed with increase in the level of stress at fifth day of treatment in all species. At 50mM increased protein was observed whereas at 100mM minor decrease was shown by all species. *S. scabra* and *S. viscosa* showed decrease in protein content at 150mM NaCl stress level. At 200mM of NaCl, *S. humilis*, *S. hamata* and *S. viscosa* showed increased protein whereas *S. scabra* and *S. seabrana* showed decreased content of protein.

#### **Malondialdehyde content (MDA)**

The salinity induced changes in the level of lipid peroxidation in terms of malondialdehyde (MDA) content was measured under both control and stress conditions. MDA content was significantly higher under salt stress. Malondialdehyde content obtained in *stylosanthes* species under salt stress significantly express the stress mechanism in a biochemical point of view. Change in level of lipid peroxidation in terms of malondialdehyde (MDA) content in five species of *Stylosanthes* is depicted in Table 4A. Increase in its level was observed in *S. hamata*, *S. scabra* and *S. humilis* at 50mM of NaCl, whereas an initial decrease in its content was observed in *S. seabrana*. Decrease in

**Table 4A.** Level of Proline, MDA and Protein content in seedlings of five species of stylo under different levels of salinity

Species	Proline (U/gm fr. wt.)							
	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	9.625	12.75	42.8	15.8	11.725	16.175	47.55	18.65
<i>S. scabra</i>	23.85	46.425	60.225	45.25	43.625	50.35	90.6	50.25
<i>S. viscosa</i>	37.775	49.475	57.75	31.75	66.85	53	92.75	67.25
<i>S. hamata</i>	17.95	41.3	52.775	58.675	21.625	58.8	86.275	78.625
<i>S. humilis</i>	34.65	45.75	39.025	33	61.95	66.25	74.5	58.75
Species	Protein (mg protein/gm fr. wt.)							
	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	22.296	34.212	32.184	30.324	22.176	44.016	42.168	53.592
<i>S. scabra</i>	33.036	34.56	35.856	30.924	25.83	30.114	33.39	26.67
<i>S. viscosa</i>	33.744	42.24	45.6	41.676	29.064	33.768	46.788	42.504
<i>S. hamata</i>	24.192	33.552	38.604	43.164	32.382	40.194	42.756	48.72
<i>S. humilis</i>	27.948	36.984	38.4	41.136	14.91	34.062	41.58	43.808
Species	MDA content (nanomoles/gm fr. wt.)							
	Third day				Fifth day			
	control	50 mM	100mM	150mM	control	50 mM	100mM	150mM
<i>S. seabrana</i>	20.98	15.73	13.35	7.22	30.69	45.33	25.89	30.58
<i>S. scabra</i>	9.43	15.96	10.08	6.14	10.45	15.86	14.36	20.47
<i>S. viscosa</i>	17.3	16.57	17.6	28.66	19.4	25.36	19.74	24.15
<i>S. hamata</i>	9.07	25.44	13.64	21.71	11.78	32.47	33.48	40.36
<i>S. humilis</i>	11.18	16.37	10.29	19.1	14.36	28.69	40.91	48.79

MDA content was noticed at 100mM of stress in all species. *S. viscosa* showed no difference in MDA activity at 100mM stress level. Further increase in level of NaCl induced increase in MDA content of *S. viscosa*, *S. hamata* and *S. humilis*. Decrease in MDA content was observed on 3<sup>rd</sup> day of salinity stress in *S. seabrana* and *S. scabra* at 150mM (Table 4A).

### Proline content

Proline was estimated using seedlings of five species of *Stylosanthes* from both control and stressed conditions. Proline was measured at third and fifth day of salt stress. Proline content increased at 50mM stress level and of the five species *S. hamata* showed maximum increase from control (17.95 to 41.30  $\mu$ moles/ gm fr.wt) (Table 4A). At 100mM of stress level proline content increased in all species, whereas at 150mM only *S. hamata* showed increase in proline content. Maximum proline content at 200mM of stress was observed in *S. hamata* (17.95 to 48.675  $\mu$ moles/ gm fr. wt). At higher level of stress proline content was observed even when seedlings were exposed to five days of stress. Maximum increase in proline content was observed in *S. scabra* at five days of stress at 150mM of stress (38.406 to 110.778  $\mu$ moles/ gm fr. wt) (Fig. 12 D). During salt stress proline content increased gradually up to 100mM stress in *Stylosanthes* seedlings while at 150mM it decreased. Maximum proline content at higher stress level was observed in *S. hamata*.

### Total osmolyte accumulation

Total osmolyte concentration (osmolality) was measured in seedlings of five species of *Stylosanthes* grown in moist petri plates with water (control) and different salt concentrations in stress conditions. Osmolyte content was initially lower in *S. viscosa* i.e., 375 mmoles/kg, whereas it was same in *S. hamata*, *S. humilis*, *S. seabrana* and *S. scabra*. In *S. seabrana* and *S. scabra* it varied from 416-862 mmoles/kg, 436-839 mmoles/kg respectively. In *S. viscosa* it showed lowest change from 375-693 mmoles/kg. *S. hamata* and *S. humilis* showed similar change in osmolality 428-922 mmoles/kg, 443-1018 mmoles/kg respectively during salt stress imposition. Osmolyte content was observed increasing with increasing concentration of NaCl (Table 4C).

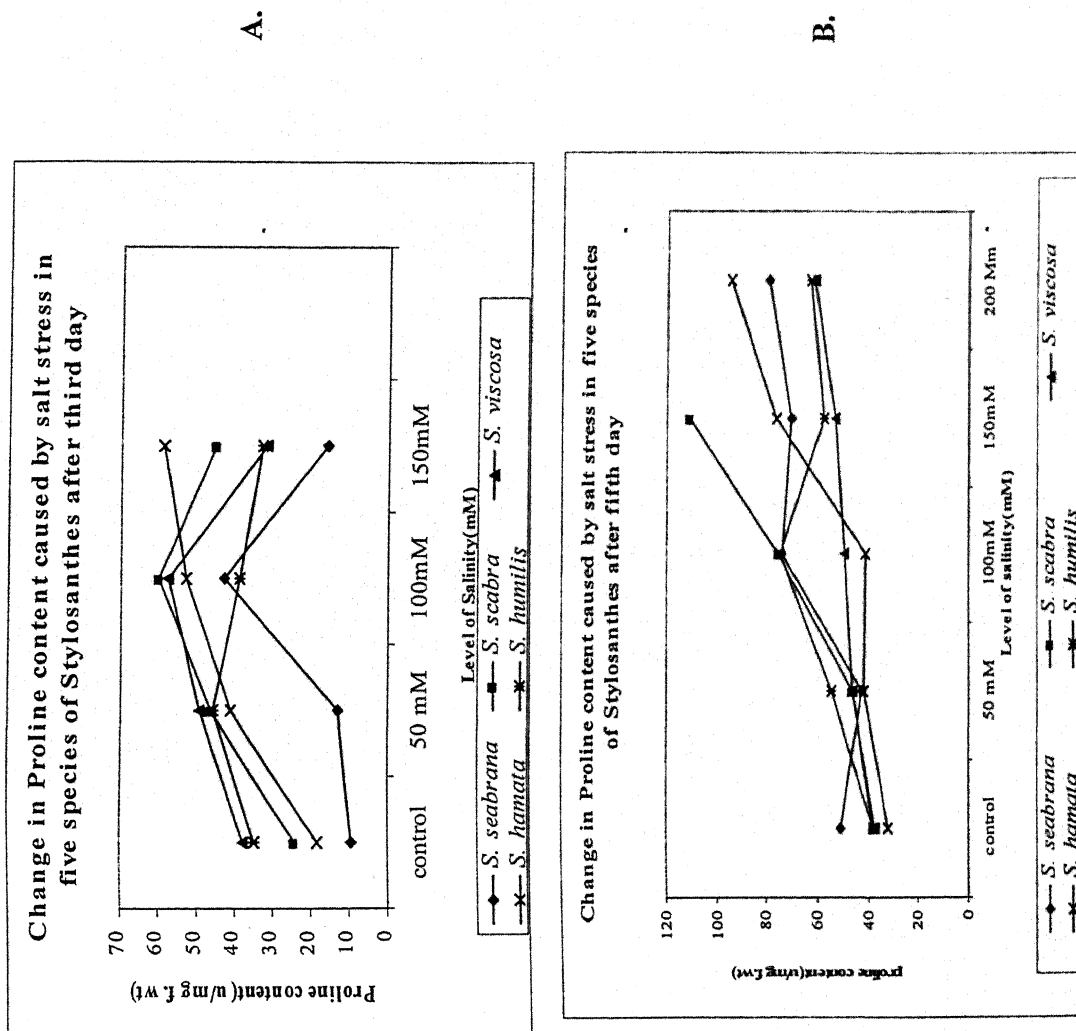


Fig 12D: Change in Proline content A. 3<sup>rd</sup> day and B Fifth day of stress in seedlings

**Table 4B.** Water potential (-MPa) in seedlings exposed to 3 days of salinity.

Species	control	50mM	100mM	150mM
<i>S. seabrana</i>	-0.89	-1.04	-1	-1.53
<i>S. scabra</i>	-0.6	-1.38	-1.73	-1.97
<i>S. viscosa</i>	-0.56	-1.07	-1.80	-1.18
<i>S. hamata</i>	-0.64	-0.87	-0.78	-1.89
<i>S. humilis</i>	-0.23	-1.33	-0.80	-1.51

**Table 4C.** Osmolality (m moles/ kg) in seedlings exposed to 3 days of salinity.

	control	50mM	100mM	150mM
<i>S. seabrana</i>	416	614	712	862
<i>S. scabra</i>	436	643	740	839
<i>S. viscosa</i>	375	600	674	692
<i>S. hamata</i>	427	615	690	922
<i>S. humilis</i>	443	580	663	1018

### Water potential

Fresh and full grown seedlings of five species of *Stylosanthes* was used for water potential experiment. For control, seeds were grown in simple water and different salt concentration was used for salt treatment. Water potential was uniformly decreased (more negative) with increasing magnitude of salt stress. Initially minimum water potential was observed in *S. humilis*. It also showed maximum change or decrease from control to stress stages. At 50mM water potential was increased (-0.2 to -1.3 MPa) but at 100mM it decreased (-1.3 to -0.8 MPa) very sharply and again at 150mM it was further increased to its higher value (-1.5 MPa). *S. hamata* showed water potential change from -0.6 to -1.8 MPa. Highest decrease in water potential was measured in *S. scabra* as compared to *S. viscosa* from -0.6 to -1.9 MPa and -0.5 to -1.1 MPa respectively. However, in *S. seabrana* water potential was change from -0.8 to -1.5 MPa. *Stylo* seedlings displayed a rapid reduction in water potential after third day of salt stress (Table 4B).

### Experiment 6:

#### Enzyme activities in leaves of *stylo* grown in sand

One week old seedlings were exposed to salinity stress of different levels. Experiment was carried out for sixty days. Solution of NaCl of the concentration of 50, 100 and 150mM was given along with Hoagland's nutrient solution once in a week in pots of *S. seabrana*, *S. scabra*, *S. viscosa*, *S. hamata* and *S. humilis*. The experiment was carried out in three replicates. Change in fresh weight and dry weight was recorded per plant basis before final harvest of the plants (Table 5A and B). In genral reduction in both fresh and dry weigh of the plant was observed. With increasing concentration of NaCl, more reduction in weight of the plant was noticed. The following enzyme activities were measured in leaves. Proline content was measured at first week and before harvesting stage of plants. Chlorophyll pigment was measured at three different stages of stress. Enzyme activities were measured in leaves before harvesting of the plants.

**Table 5A.** Fresh weight (gm/plant) of plants grown under different levels of salinity of five species of stylo.

<i>S. seabrana</i>	Root	Shoot	<i>S. scabra</i>	Root	Shoot
control	0.59433333	2.29983333	control	1.1533333	4.66
50 mM	0.65833333	1.92466667	50 mM	2.9466667	3.626
100mM	0.70666667	1.40666667	100mM	1.3058333	2.804
150mM	0.99	1.605	150mM	3.67	4.1883333

<i>S. viscosa</i>	Root	Shoot	<i>S. hamata</i>	Root	Shoot
control	1.41933333	4.61833333	control	1.56083333	4.86166667
50 mM	1.544	4.585	50 mM	3.90666667	4.17833333
100mM	2.44766667	3.11833333	100mM	1.85666667	3.55483333
150mM	1.27633333	3.795	150mM	2.87983333	3.00166667

<i>S. humilis</i>	Root	Shoot
control	0.79266667	2.659
50 mM	2.64333333	4.83666667
100mM	1.74333333	4.56166667
150mM	2.084	2.76

**Table 5B.** Dry weight (gm/plant) of plants grown under different levels of salinity of five species of stylo.

<i>S. seabrana</i>	Root	Shoot	<i>S. scabra</i>	Root	Shoot
control	0.321	0.959	control	0.649	1.62
50 mM	0.303	0.810	50 mM	0.498	1.48
100mM	0.276	0.566	100mM	0.354	0.815
150mM	0.237	0.690	150mM	0.784	1.42

<i>S. viscosa</i>	Root	Shoot	<i>S. hamata</i>	Root	Shoot
control	0.359	1.30	control	0.919	2.15
50 mM	0.387	1.24	50 mM	0.926	1.88
100mM	0.327	1.17	100mM	0.632	1.35
150mM	0.307	1.14	150mM	0.486	1.58

<i>S. humilis</i>	Root	Shoot
control	0.549	1.33
50 mM	0.815	1.89
100mM	0.466	1.79
150mM	0.312	1.44

### Glycolate oxidase (GO)

No significant change in glycolate oxidase activity was observed in *Stylo* species except *S. scabra*. Increase in activity was noticed in *S. scabra* at 50mM of stress level which decreased when salinity level increased (Fig. 13B). *S. hamata* and *S. humilis* showed maximum decrease in activity whereas *S. seabrana*, *S. scabra* and *S. viscosa* showed minor increase in activity at higher levels of stress. At 150mM of NaCl stress, *S. hamata* showed increased glycolate oxidase activity as compared to other species (Table 6).

### Catalase (CAT)

Catalase activity was estimated in leaves of *S. seabrana*, *S. scabra*, *S. viscosa*, *S. hamata* and *S. humilis* species of *Stylosanthes* before final harvest of plants. Catalase activity in *S. hamata*, *S. seabrana* and *S. humilis* was high to that of *S. scabra* and *S. viscosa* in control plants (Fig. 13A). However, activity declined with the increase of stress level. *S. scabra* showed increase in activity at lower stress level but significantly decreased at 100mM of stress. No significant variations were observed in activity of *S. viscosa* in control and stress conditions. Maximum CAT activity was observed in *S. seabrana* (1250 u/mg f.wt) at the highest level of stress and minimum in *S. viscosa* (150u/mg f.wt) (Table 6).

### Peroxidase (PRX)

Peroxidase activity increased in *S. seabrana* and *S. viscosa* and decreased in *S. hamata*, *S. humilis* and *S. scabra*. Mosaic patterns of peroxidase activity was shown by five species of *Stylosanthes* at minimal level of stress. *S. hamata*, *S. humilis* and *S. scabra* showed increase in activity at 50mM stress which further decreased and finally increased at higher stress level (Fig 13 A). At the final stage of harvest all species showed increase in peroxidase activity. The increase in activity in *S. humilis* was minimum (666.67 to 981.67 unit/mg f.wt) whereas in *S. hamata*, it was maximum (756-1676.67 unit/mg f.wt.) (Table 6).

**Table 6.** Enzyme activities measured in leaves of stylo species grown under salinity (60 days). Activity measured before final harvest of the crop.

	Peroxidase (U/gm fr. wt./min)				Ascorbate peroxidase (U/gm fr.wt./min)			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	1055	775	998.3	796.67	58750	45000	45000	51250
<i>S. scabra</i>	188.3	515	161.6	490	40000	81250	32500	37500
<i>S. viscosa</i>	348.3	175	225	823.33	43750	26250	26250	15000
<i>S. hamata</i>	756	988.3	506.67	1676.67	137500	115000	51250	77500
<i>S. humilis</i>	666.67	1091.67	660	981.67	101250	68750	68750	55000

	Superoxide dismutase (U/gm fr. wt./min)				Glutathione reductase (U/gm fr.wt./min)			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	133.12	115.31	129.06	123.75	4700	5800	5500	3920
<i>S. scabra</i>	119.06	115.62	111.25	125.93	5800	5920	5160	3300
<i>S. viscosa</i>	108.75	107.81	104.56	115	5720	6120	3240	2480
<i>S. hamata</i>	124.37	115.62	125.62	118.43	6200	5620	5000	3600
<i>S. humilis</i>	99.68	104.37	110.93	92.5	5920	6960	5800	2140

	Catalase before harvest (u/gm fr.wt./min)				Glycolate oxidase (OD/gm fr. wt./min)			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	2900	1350	3500	4550	10.296	8.679	10.098	10.494
<i>S. scabra</i>	150	1000	350	1250	7.557	9.669	6.963	7.425
<i>S. viscosa</i>	150	400	400	650	8.019	7.095	7.194	7.392
<i>S. hamata</i>	3150	1100	1650	2550	12.177	9.141	7.524	9.108
<i>S. humilis</i>	2450	1050	1050	1450	8.976	8.019	7.623	6.93

### Ascorbate peroxidase (APX)

Ascorbate peroxidase activity increased in *S. seabrana*, *S. viscosa*, *S. hamata* and *S. humilis* whereas decreased in *S. scabra*. The minimum activity was found in control plants of *S. scabra* (40000 unit/mg fr. wt.), whereas maximum in *S. hamata* (137500 unit/mg f.wt.) (Fig. 13B). *S. scabra* showed different patterns as compared to other species when level of stress was increased. *S. hamata*, *S. humilis*, *S. seabrana* and *S. viscosa* showed initial decrease in activity of APX at stress level of 50mM. Even after higher level of stress APX activity decreased (Table 6).

### Superoxide dismutase (SOD)

Superoxide dismutase enzyme activity was measured in five species of *stylosanthes* under control and salt stress condition and represented as units/ mg fr. wt. min<sup>-1</sup>. In general similar patterns of SOD activity was observed before final harvest. At 50mM stress level minor decrease in SOD activity was observed in all species. *S. seabrana* showed maximum increase in activity at the stress level of 100mM. Minimum SOD activity at 100mM stress level was noticed in *S. viscosa*. *S. scabra* and *S. viscosa* showed decrease SOD activity at 100mM NaCl stress, whereas *S. seabrana*, *S. hamata* and *S. humilis* showed increased activity (Fig 13 A). SOD activity at 150mm of stress showed decreased in all species of *Stylosanthes* (Table 6).

### Glutathione reductase (GR)

Glutathione reductase activity at the harvesting stage of plants of *Stylosanthes* showed initial increase in its activity at 50mM of NaCl stress. At 100mM all species showed decreasing trend in activity. *S. viscosa* showed maximum decrease in activity at 100mM level of stress (Fig. 13A). At highest stress level decreased activity was shown by all species. *S. humilis* showed maximum decrease in glutathione reductase activity at 150mM of NaCl stress (Table 6).

### Chlorophyll pigments

Chlorophyll content was estimated at different days of stress in five species of *Stylosanthes* in first week of salt treatment. Chlorophyll a content was observed more at

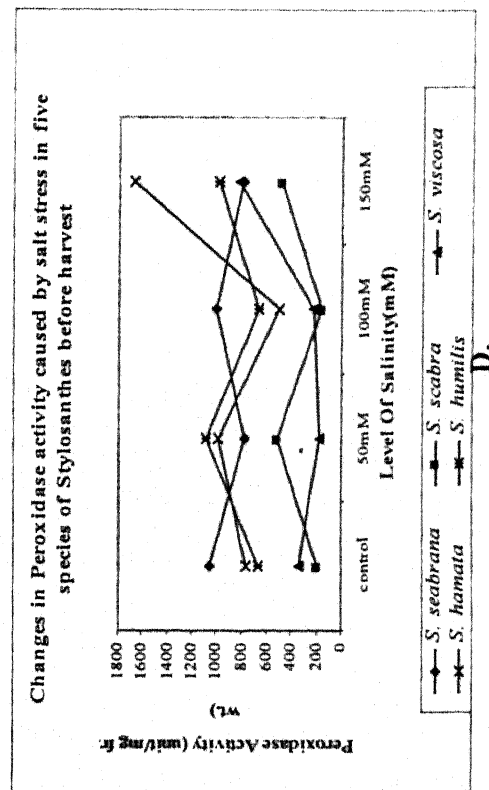
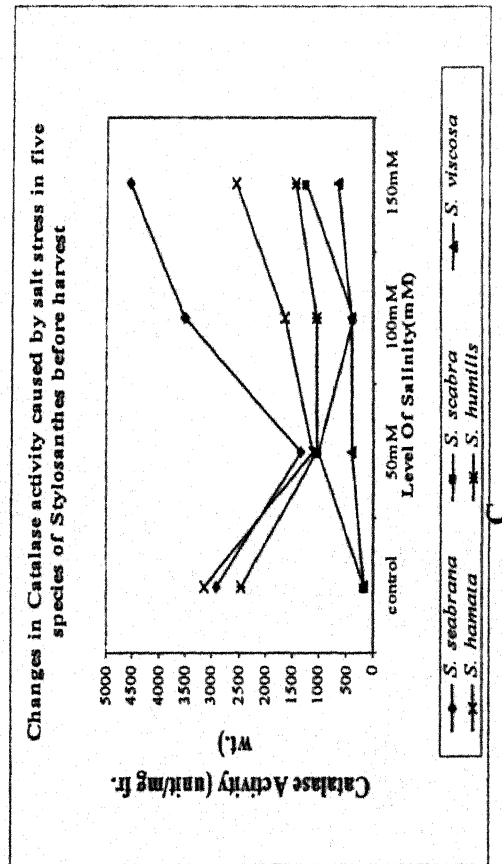
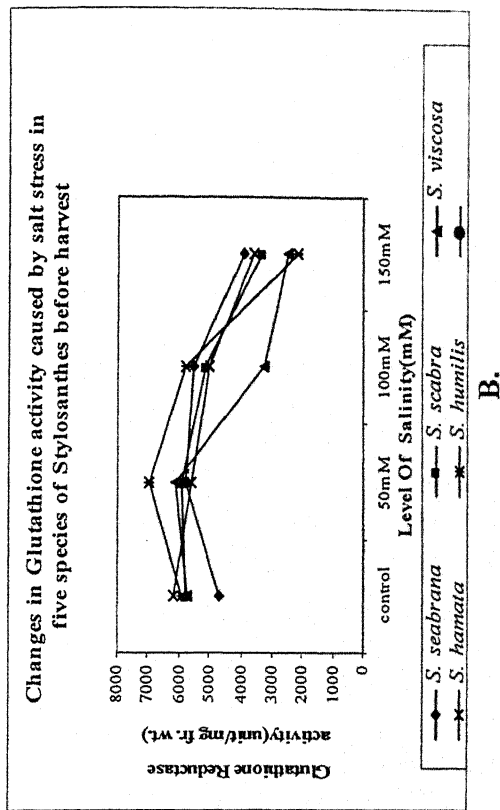
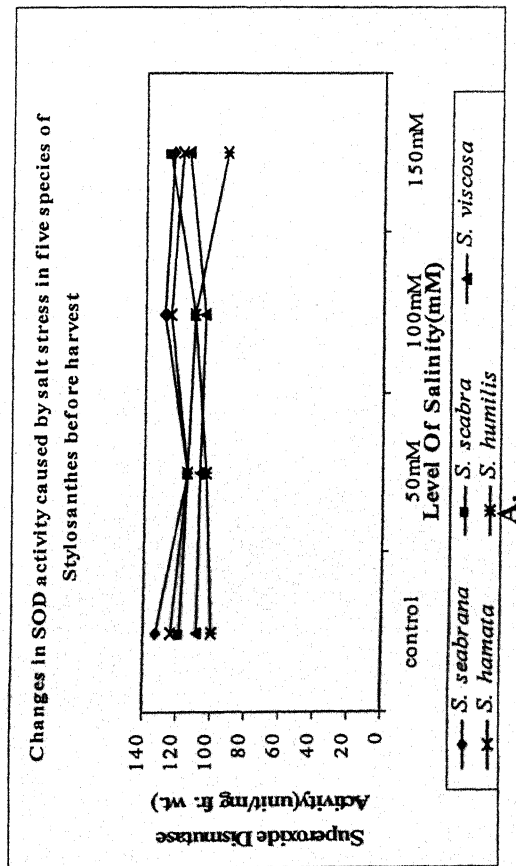
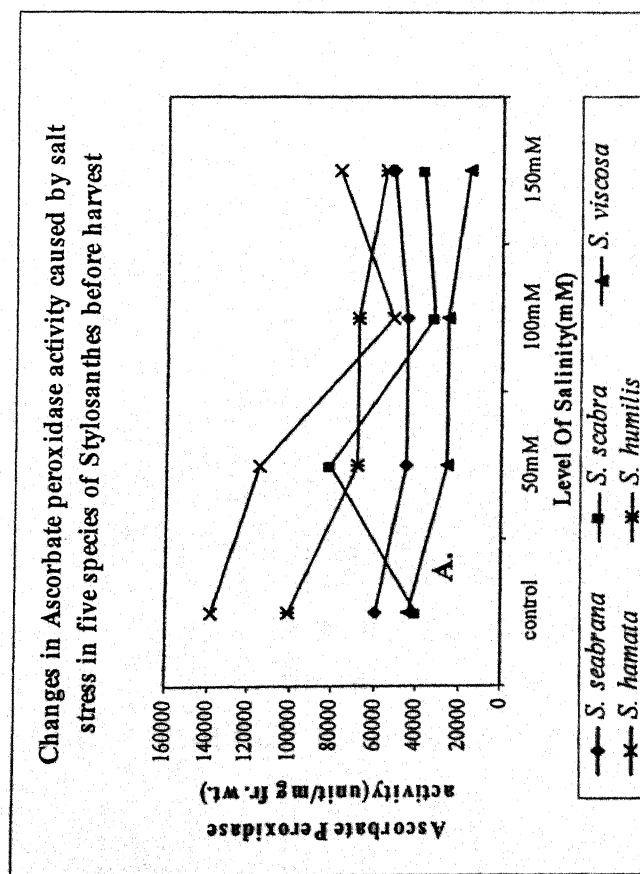
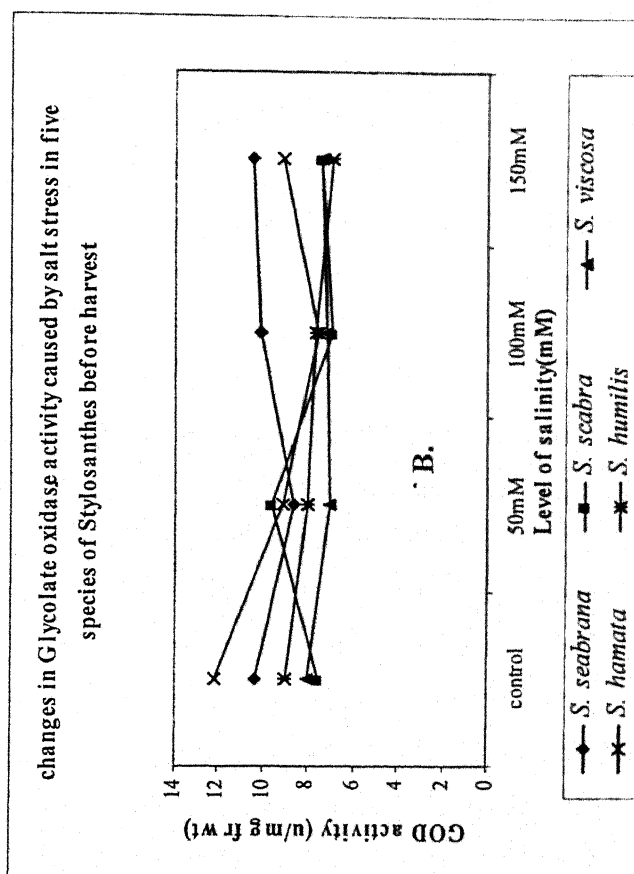


Fig 13A: Change in A. SOD, B. Glutathione reductase C. Catalase D. Peroxidase activity before harvest in leaves of plants subjected to log duration of salt stress..



A.



B.

Fig 13B: Change in A. Ascorbate peroxidase activity B. Glycolate oxidase activity in leaves of plants subjected to log duration of salt stress..

50mM salinity stress to that of control of each species. Chlorophyll content declined with the increase of stress. No significant variations in content of chlorophyll a was observed in all species in control condition, whereas at highest stress level maximum chlorophyll a content was observed in *S. scabra* (3.33mg/gm f.wt) and minimum in *S. seabrana* (1.28 mg/gm f.wt) (Fig. 14 A, Table 8). In general chlorophyll a content increased with stress in the first week of treatment.

Variations in terms of increase and decrease in chlorophyll a content was observed when measured after 4 weeks of treatment in five species of *Stylosanthes*. Four Species namely *S. scabra*, *S. seabrana*, *S. viscosa* and *S. humilis* showed decrease in chlorophyll a content at lower stress level. At higher stress, *S. scabra*, *S. seabrana* and *S. humilis* showed increase in chlorophyll a content whereas *S. viscosa* showed significant decrease in chlorophyll a content. *S. hamata* showed increasing trend from low to higher stress level. At the stage of harvest, all species showed decline in chlorophyll a content. Similar to chlorophyll a, chlorophyll b also increased with the increase of salt stress in five species of *Stylosanthes* in first week of treatment. In control content of chlorophyll b was similar in all species. At highest salt stress level maximum chlorophyll b content was observed in *S. scabra* (1.52 mg/gm f.wt) and minimum in *S. seabrana* (0.287 mg/gm fr. wt). The level of chlorophyll b ranged from 0.287 to 1.52 mg/gm fr. Wt (Fig. 14B, Table 8).

At four weeks of stress, except *S. hamata* all other species showed initial decrease at lower stress level and then increased at higher stress level. From the beginning of stress or 50mM NaCl level, *S. hamata* showed increase content of chlorophyll b even at higher stress level. Increased chlorophyll b content was observed in all species at 150mM of NaCl stress. At the stage of final harvesting, *S. hamata* and *S. viscosa* showed increased chlorophyll b content. Significant decrease was observed in chlorophyll b content of *S. humilis*. Carotene content was also increased upon stress exposure in all species of *Stylosanthes* in first week of treatment (Table 8). The maximum value was observed in *S. scabra* in both control and stressed plants. *S. humilis* showed maximum increase in carotene at stress 50mM but at higher stress level carotene content decreased. After 4

**Table 8.** Chlorophyll and carotene content determined at different stages of salinity stress in five species of stylo.

Species	Chlorophyll a after one week				Chlorophyll a after mid of treatment			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	1.29	1.52	1.41	1.28	0.944	1.07	1.12	2.22
<i>S. scabra</i>	1.94	1.42	1.24	3.33	1.35	1.28	0.867	2.24
<i>S. viscosa</i>	1.084	1.49	1.118	1.77	1.118	0.937	1.193	0.266
<i>S. hamata</i>	1.534	1.264	1.287	2.059	1.377	1.673	1.9	1.986
<i>S. humilis</i>	1.73	2.31	1.23	2.03	1.006	0.895	1.346	1.447

	Chlorophyll a before harvest				Chlorophyll b before harvest			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	1.477	0.906	1	0.839	0.368	0.244	0.32	0.274
<i>S. scabra</i>	1.049	1.22	0.545	0.743	0.267	0.305	0.248	0.286
<i>S. viscosa</i>	0.958	0.845	0.457	0.534	0.353	0.249	0.209	0.349
<i>S. hamata</i>	1.47	1.22	1.388	1.061	0.392	0.362	0.407	0.45
<i>S. humilis</i>	1.449	0.823	0.647	0.894	0.364	0.295	0.252	0.107

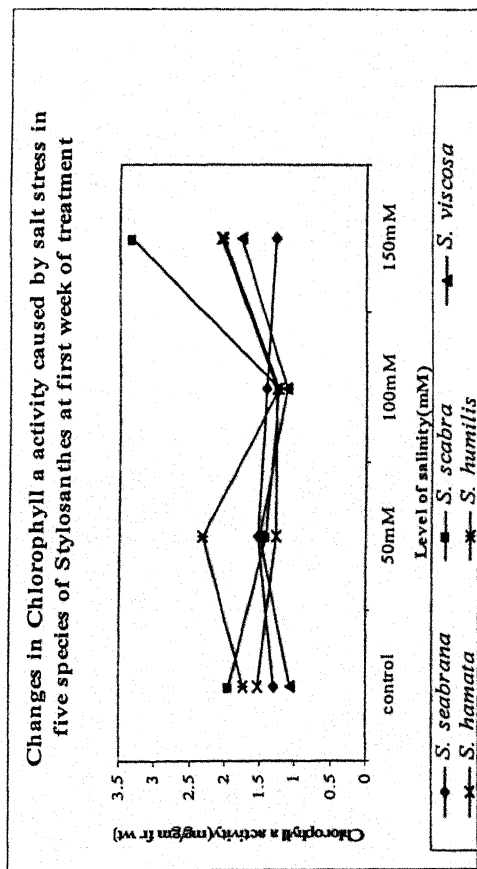
	Chlorophyll b after one week				Chlorophyll b after mid of treatment			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	0.249	0.262	0.272	0.287	0.434	0.42	0.464	0.79
<i>S. scabra</i>	0.333	0.24	0.271	1.52	0.59	0.499	0.372	0.775
<i>S. viscosa</i>	0.212	0.307	0.233	0.415	0.453	0.432	0.442	0.501
<i>S. hamata</i>	0.256	0.262	0.244	0.504	0.48	0.626	0.66	0.737
<i>S. humilis</i>	0.345	0.447	0.253	0.469	0.452	0.353	0.487	0.525

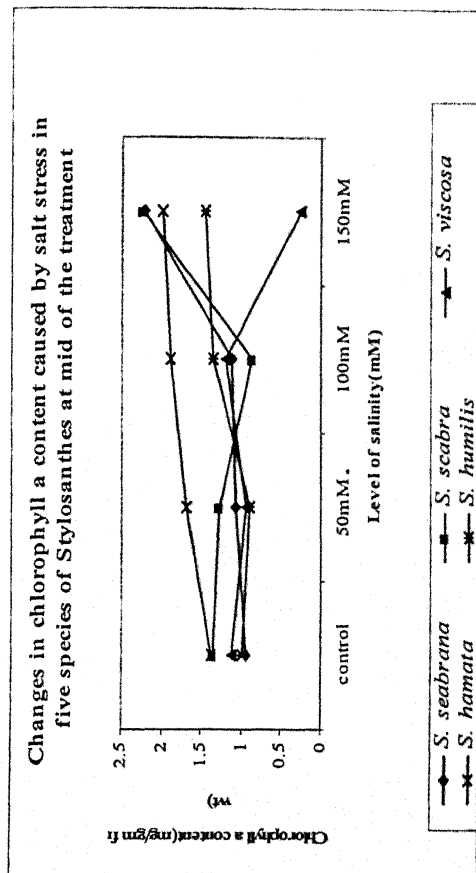
	Carotene after one week				Carotene after mid of treatment			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	2.83	3.03	3.13	2.94	2.3	2.53	2.63	4.68
<i>S. scabra</i>	3.65	3.15	2.17	6.57	2.87	2.83	2.003	4.27
<i>S. viscosa</i>	2.25	1.97	2.09	3.23	2.52	2.23	2.51	3.7
<i>S. hamata</i>	3.11	2.9	2.86	3.89	2.87	3.54	3.95	4.22
<i>S. humilis</i>	3.5	5.62	2.31	4.09	2.42	2.14	3.04	3.05

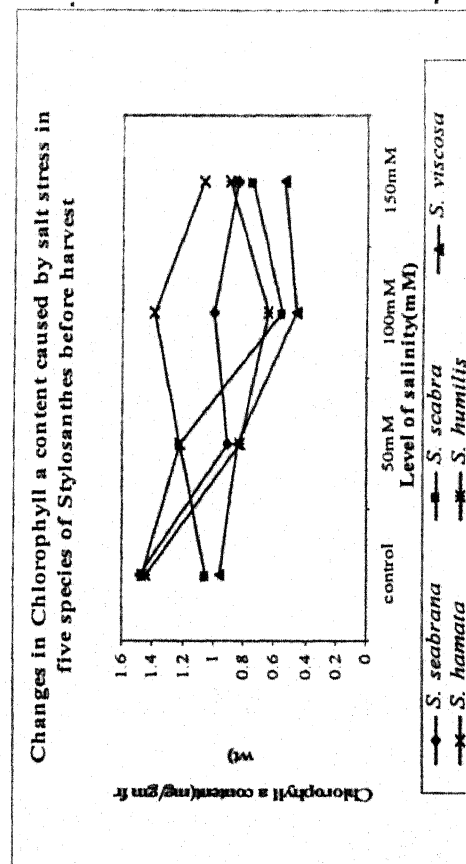
Carotene before harvest				
	control	50mM	100mM	150mM
<i>S. seabrana</i>	3.55	2.4	2.46	1.97
<i>S. scabra</i>	2.12	2.6	2.02	1.5
<i>S. viscosa</i>	2.46	2.19	1.52	1.55
<i>S. hamata</i>	2.8	2.81	2.81	2.301
<i>S. humilis</i>	3.43	2.14	1.73	2.03



A.

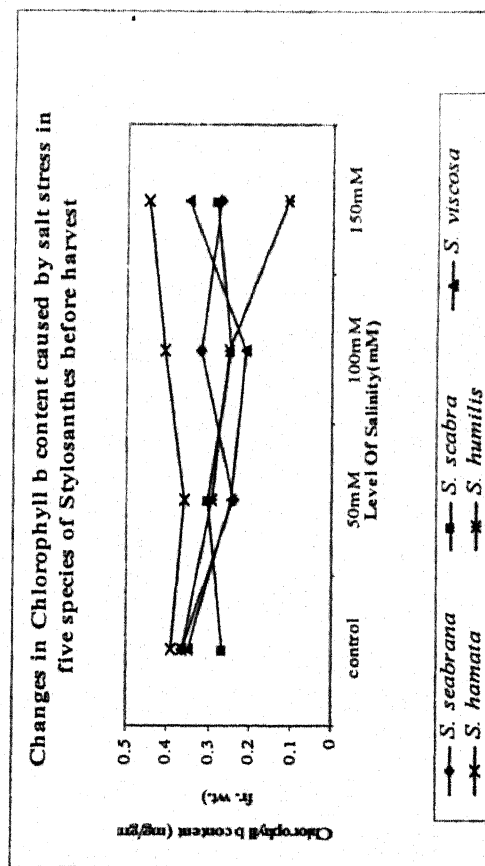
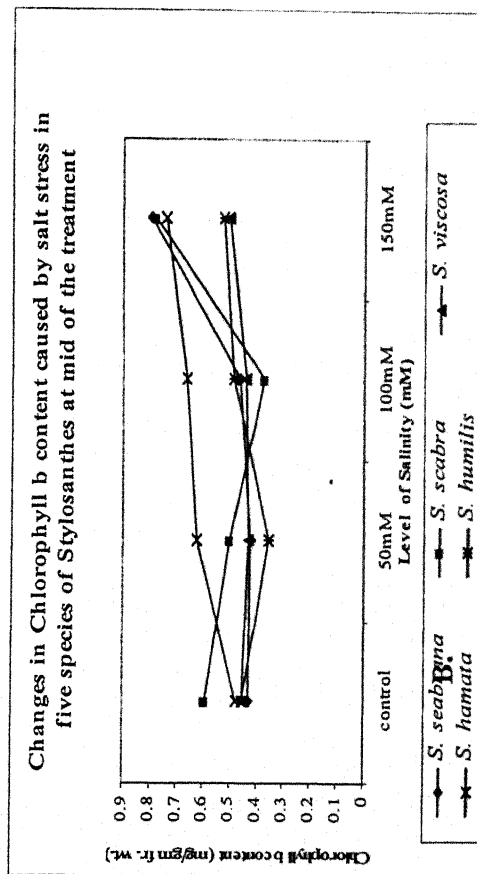
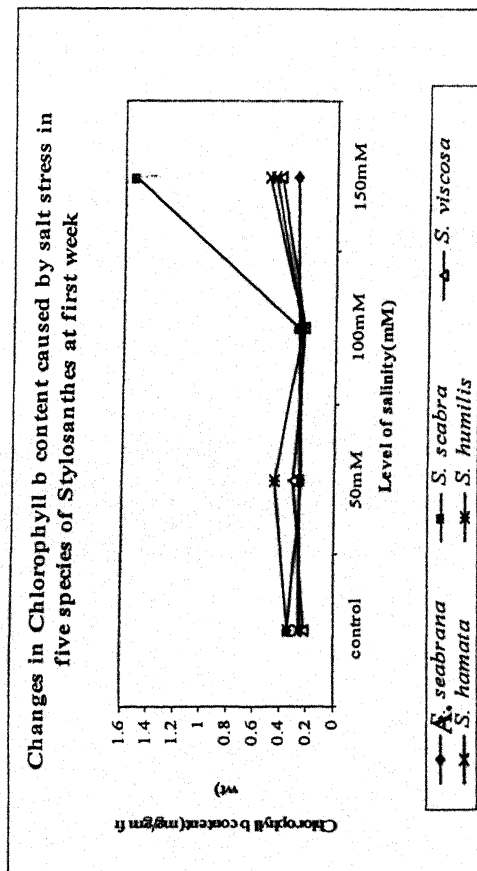


B.



C.

Fig 14A: Effect of secondary salinization on chlorophyll a pigment A. first week, B. Mid, C. Before harvest in leaves



C.

Fig 14B: Effect of secondary salinization on chlorophyll b pigment A. first week , B. Mid , C. Before harvest in leaves

weeks of treatment increasing trend was observed in all species along with the increase in stress level. *S. hamata* showed linear increase in carotene with stress level. All other species also showed increase in carotene at higher stress level. At the final harvest stage decreasing trend was observed in all species when the level of stress was increased. *S. seabrana* and *S. humilis* showed maximum decrease in carotene at highest stress level in comparison to control plants. Minimum decrease was observed in *S. hamata*. At 150mM stress level maximum carotene was observed in *S. hamata*, however in control plants minimum carotene was observed in *S. scabra* (Fig 14 C).

### **Proline content**

Proline was estimated using fresh leaf tissues from both control and stressed plants of five species of *Stylosanthes*. Proline content observed at different days of stress indicated that except *S. scabra* other species of *Stylosanthes* showed increase in proline content at 50mM of NaCl stress. Proline content was decreased in *S. scabra* at lower stress level which is increased significantly at 150mM stress. Proline content was increased in all other species at 150mM but maximum increase was observed in *S. scabra* (1.81 to 5.92  $\mu\text{mole/gm f.wt.}$ ) (Fig. 13C). However, before final harvest of the plant proline content in *S. hamata* was maximum (4.9 to 9.4  $\mu\text{mole/gm f.wt.}$ ) to that of other species (Table 7). At this stage minor increase in proline content was observed at 50mM of stress in *S. scabra*, *S. humilis* and *S. seabrana*. *S. viscosa* showed increase in activity at lower stress level which decreased significantly at 150mM of stress level.

### **Genomic DNA from stylo:**

Genomic DNA was isolated from whole young seedlings of 23 accessions of *Stylosanthes*, comprising 19 accessions of *S. seabrana*, 2 each of *S. scabra* and *S. viscosa*. Buffer 'S' (100 mM Tris-HCL pH 8.0, 50 mM EDTA, 100 mM NaCl and 2 % SDS) was used in 1:2 ratios for the grinding of seedlings. The quantity and quality of DNA was checked on 0.8 % agarose gel in 0.5 %TBE buffer and finally DNA was

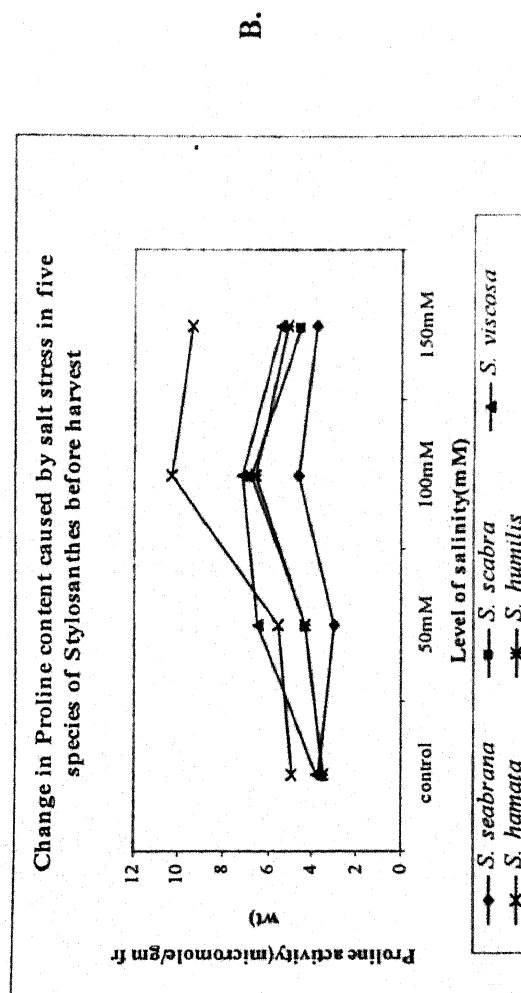
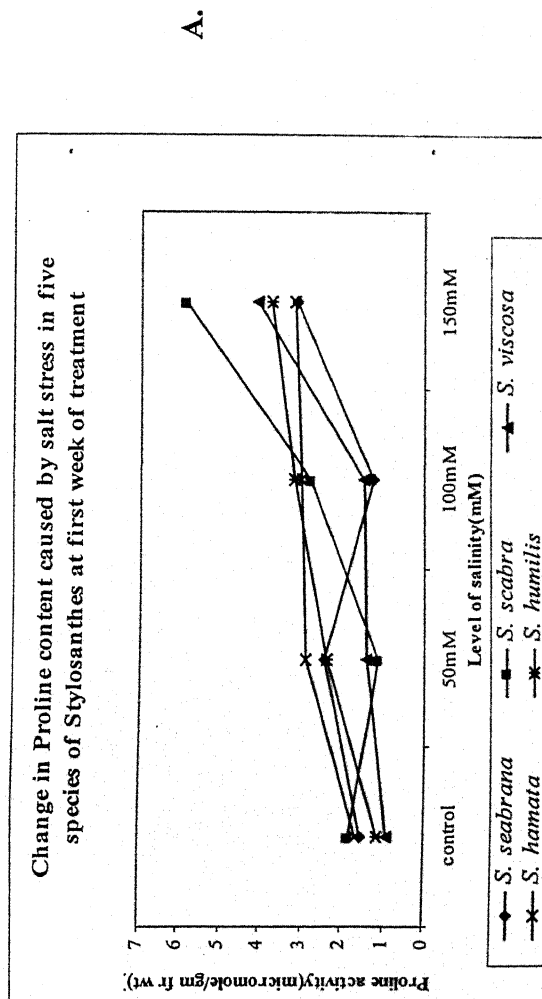


Fig 13C: Change in Proline content A. First week B. Before harvest of long duration stressed plants

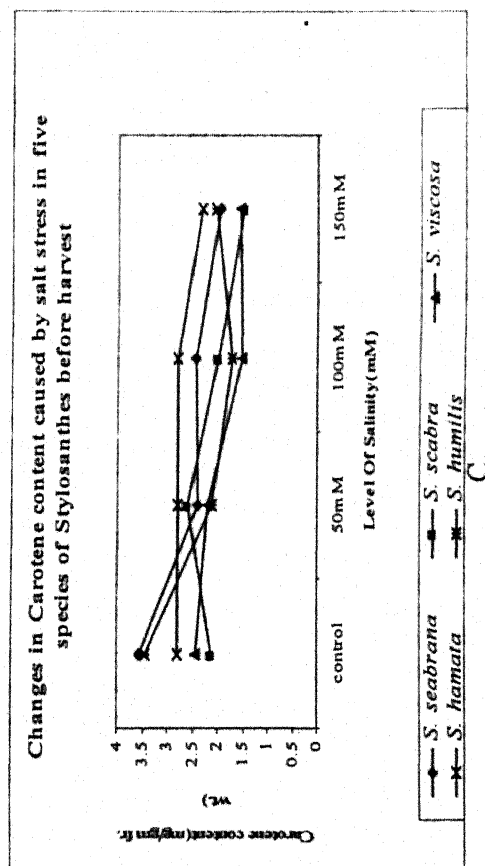
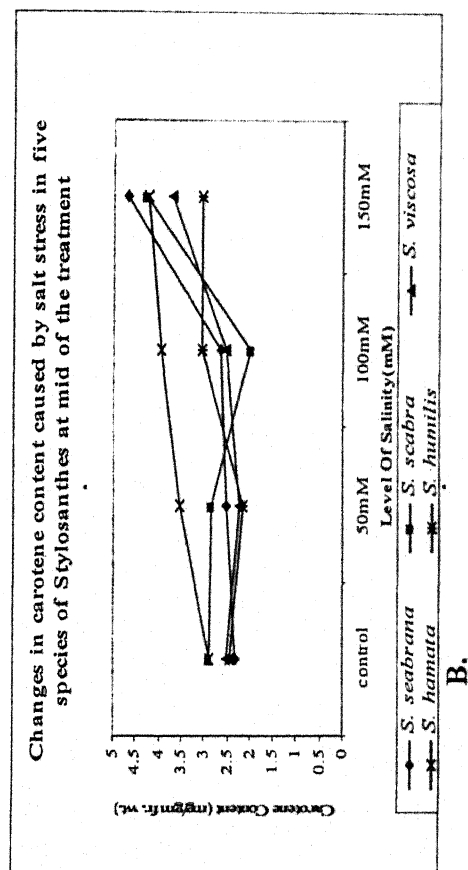
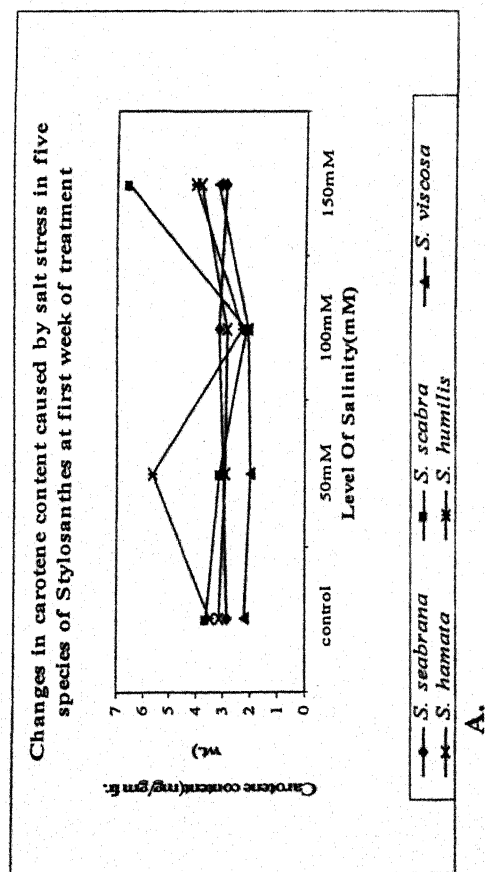


Fig 14C: Effect of secondary salinization on carotene pigment A. first week, B. Mid, C. Before harvest in leaves

**Table 7.** Level of Proline measured in leaves of long duration stress experiment at two different stages.

Species	Proline after one week ( $\mu\text{mole/gm fr. wt./min}$ )				Proline before harvest ( $\mu\text{mole/gm fr. wt./min}$ )			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
<i>S. seabrana</i>	1.56	2.49	1.25	3.16	3.7	3.04	4.63	3.86
<i>S. scabra</i>	1.81	1.13	2.82	5.92	3.55	4.31	6.725	4.5
<i>S. viscosa</i>	0.94	1.42	1.49	4.15	3.8	6.45	7.141	5.41
<i>S. hamata</i>	1.65	2.91	3.06	3.22	4.98	5.5	10.249	9.4
<i>S. humilis</i>	1.15	2.4	3.24	3.77	3.5	4.29	6.52	5.175

diluted with TE to a concentration of 5ng/μl for STS and RAPD analysis. No degradation and shearing of DNA was observed when DNA was isolated using buffer 'S'.

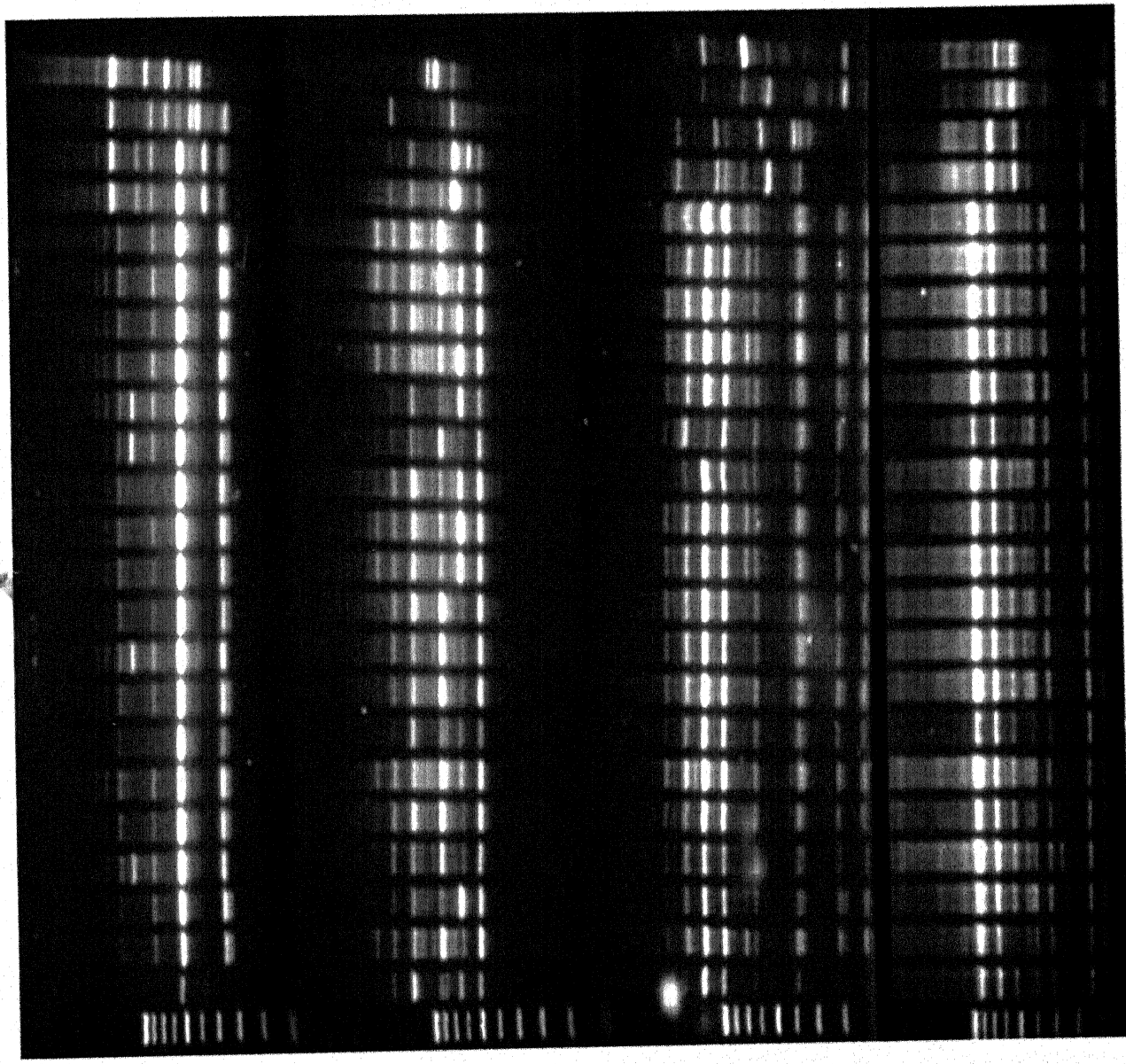
### RAPD analysis

Polymerase chain reaction (PCR) amplification was performed in a final volume of 20μl reaction mixtures containing 67mM Tris-HCL (pH 8.0), 16.6 mM ammonium sulphate, 0.45 % w/v BSA, 3.5 mM MgCl<sub>2</sub>, 150μM of each of dATP, dCTP, dTTP and dGTP, 7.5 pmol (15ng) primer, 25 ng genomic DNA template and 0.5 units Taq polymerase. Reaction mixture was overlaid with mineral oil to resist the evaporation of mixture. After amplification PCR products were run on 1.6% agarose gel running in 0.5X TBE buffer and visualized by staining with ethidium bromide. Gel was visualized in gel documentation system under UV light. In RAPD patterns observed on the gel showed distinct bands of the sizes of 200bp to 1.5Kb. Sometimes more than 2Kb fragment was also observed. In total 32 RAPD primers generated 476 DNA fragments when employed with 19 accessions of *S. seabrana* and two each of *S. scabra* and *S. viscosa*. These 32 primers have been reported to perform good reactions with *Stylosanthes* DNA (Liu, 1997). The maximum number of bands observed was 25 with primer OPR-08 (Fig. 15A and B). The percentage of polymorphism ranged from 63.64 to 100 among these 23 lines of *Stylosanthes* and polymorphism was considered by taking the bands in any one of the accessions of *S. seabrana*. The polymorphic information content and marker index as presented in (Table 9) shows significant variations among the primers selected for DNA amplification. The marker index, which indicates the polymorphic information content of the primers and number of polymorphic bands amplified by the primers, was observed as high as 11.04 indicated suitability of RAPD in genetic analysis of *Stylosanthes* species (Table 9). The sharing of RAPD markers between *S. seabrana* and *S. scabra* was significantly high (57 %) than those between *S. scabra* and *S. viscosa* (37 %) (Table 10). The proportion of RAPD bands shared between *S. scabra* and *S. seabrana* was significantly high (40 %) indicating more closeness of *S. seabrana* to the *S. scabra*. Even bands shared by three species (47) were low as compared to that of *S. seabrana* and *S. scabra* (57). The unique bands which was counted on the basis of presence of bands in all

Table 9. Details of the primers, total number of polymorphic bands, percentage of polymorphism, PIC and MI value

RAPD primers	Total no. of bands	Total no. of polymorphic bands	% of polymorphic bands	PIC	MI
OPQ-05	12	10	83.33	0.225	2.25
OPT-06	18	18	100	0.47	8.49
OPQ-09	13	12	92.30	0.49	5.88
OPQ-03	17	17	100	0.49	8.33
OPR-04	20	19	95	0.23	4.44
OPR-08	25	24	96	0.46	11.04
OPR-07	15	13	86.66	0.48	6.24
OPN-04	11	7	63.63	0.37	2.6
OPN-05	13	12	92.30	0.49	5.88
OPN-06	18	17	94.44	0.49	8.33
OPAH-01	13	11	84.61	0.49	5.39
OPAH-03	10	8	80	0.44	3.56
OPAH-08	20	20	100	0.49	9.9
OPAF-13	17	15	88.23	0.49	7.4
OPP-09	16	12	75	0.49	5.88
OPAE-01	23	20	86.95	0.49	9.86
OPAE-02	11	11	100	0.49	5.39
OPAE-03	21	20	95.23	0.43	8.6
OPAE-07	17	15	88.23	0.49	7.35
OPG-05	7	7	100	0.48	3.36
OPG-10	18	17	94.44	0.49	8.48
OPG-15	8	8	100	0.38	3.10
OPI-16	8	8	100	0.48	3.89
OPJ-13	20	14	70	0.46	6.44
OPE-11	6	5	83.33	0.50	2.52
OPF-06	14	12	85.71	0.49	5.88
OPAB-01	15	10	66.66	0.348	3.48
OPAB-03	14	12	85.71	0.49	5.95
OPAB-05	6	5	83.33	0.5	2.5
OPA-08	17	17	100	0.49	8.33
OPAK-18	18	17	94.44	0.49	8.43
OPAK-20	15	15	100	0.49	7.42
Total/Mean	476/14.875	418/13.06	2865.2/89.5	14.49/0.45	196.59/6.14

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23



**Fig 15A: PCR patterns with RAPD primer (A)OPAE 07 (B)OPF 06 (C)OPAH 08 (D)OPN 06 (top to bottom). Lanes 1-19 *S. scabrona*, Lanes 20-21 *S. scabra*, lanes 22-23 *S. viscosa*, M = 100 bp ladder**

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

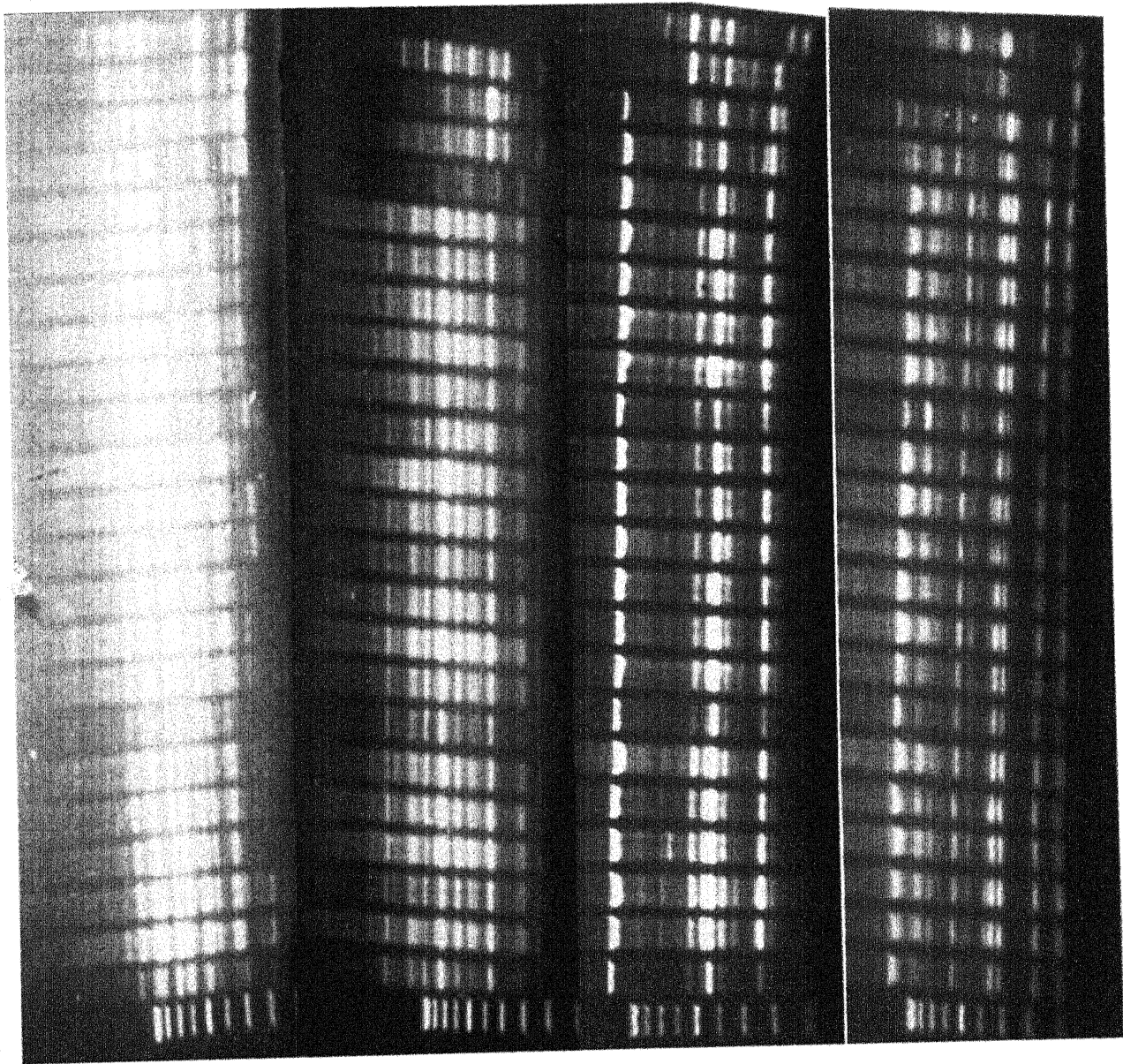


Fig 15B: PCR patterns with RAPD primers OPAB 01, OPAE 01, OPJ 13 and OPT 06 (top to bottom). Lanes 1-19 *S. scaberrima*, Lanes 20-23 *S. viscosa*, lanes 22-23 *S. viscosa*, M = 100 bp ladder



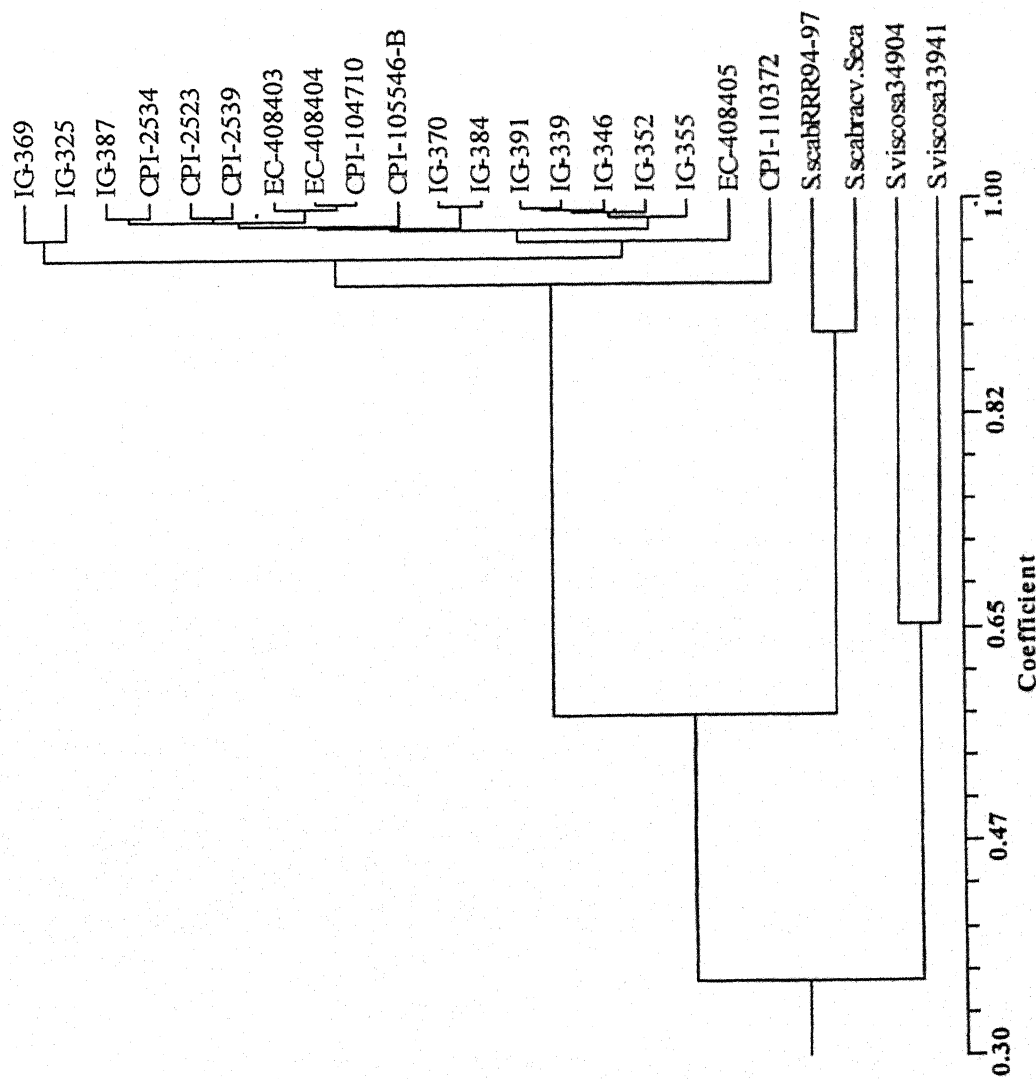


Fig 15C: Dendrogram showing genetic relationship among five species of stylo comprising 23 accessions as revealed by DNA markers based on UPGMA of DICE similarity coefficients and SAHN clustering.

accessions of a species was high in *S. seabrana* as compared to *S. scabra* and *S. viscosa* indicated more closeness of *S. seabrana* among themselves.

Dendrogram analysis of RAPD bands present or pair wise genetic distance based on dice similarity coefficient of 476 RAPD bands revealed two main clusters in UPGMA module. Dendrogram revealed two major clusters namely cluster I and cluster II (Fig. 15C). These two clusters show 35% genetic similarity to each other. Cluster I was further divided into subclusters, subcluster I and subcluster II. These two subclusters showed 57% genetic similarity. Subcluster I embodied nineteen *S. seabrana* accessions while cluster II consisted two *S. scabra* accessions. Cluster II embodied two accessions of *S. viscosa*. Among two *S. viscosa* accessions 61% genetic similarity was observed. Subcluster I of cluster I was further subdivided in two sus-sub-clusters at 92% genetic similarity level wherein 18 accessions of *S. seabrana* formed are clustered and one accession CPI 110372 as a distinct group. Subcluster II embodied two *S. scabra* accessions at 59% genetic similarity with sub-sub-cluster. These two accessions of *S. scabra* revealed 90% similarity among them. Two accessions of *S. seabrana* namely IG 369, and IG 325 formed distinct group to that of rest of 17 *S. seabrana* accessions. Among nineteen accessions of *S. seabrana* accessions EC 408404 and CPI 104710 showed 100% genetic similarity. Similarly IG 391 and IG 339 were 100% similar.

### STS analysis

For STS-PCR, 17 STS primer pairs used in the present investigation were primarily derived from the *Pst*I clones obtained from *S. scabra* cv Fitzroy and *S. hamata* cv Verano (Vander Stappen *et al.*, 1999) as well as on different coding and non-coding regions of gene sequences (Curtis *et al.*, 1995; Liu *et al.*, 1996; Manners *et al.*, 1995; Reddy *et al.*, 1996; Smith *et al.*, 1995) (Table 11). The OLIGO (version 3.0) computer program was used to select the optimal oligonucleotide for STS primer pairs as described by Liu *et al.* (1996). An oligonucleotide was selected only when its  $T_m$  was greater than 50 °C and when its 3' terminus was not complementary to itself or to the other primer with which they form a pair. Primers were synthesized by Sigma Genosys Company

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

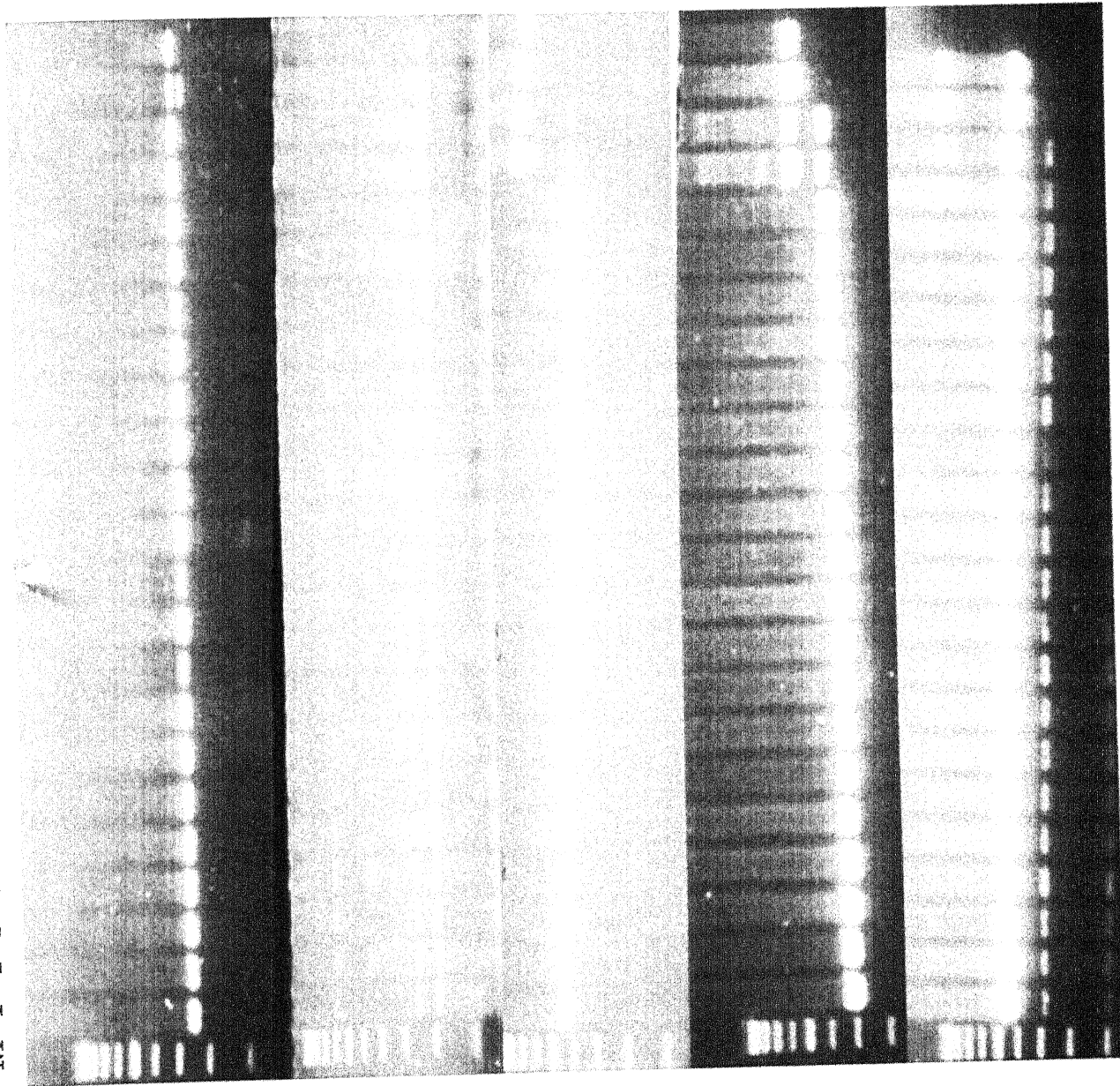


Fig. 16A: PCR patterns with STS primers 284P1/P2, AF1/AR12, T2F3/T2R18, AF1/AR1 and T2F3/T2R16 (top to bottom). Lanes 1-19 *S. scabrous*, Lanes 20-21 *S. viscosa*, lanes 22-23 *S. viscosa*, M = 100 bp ladder

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

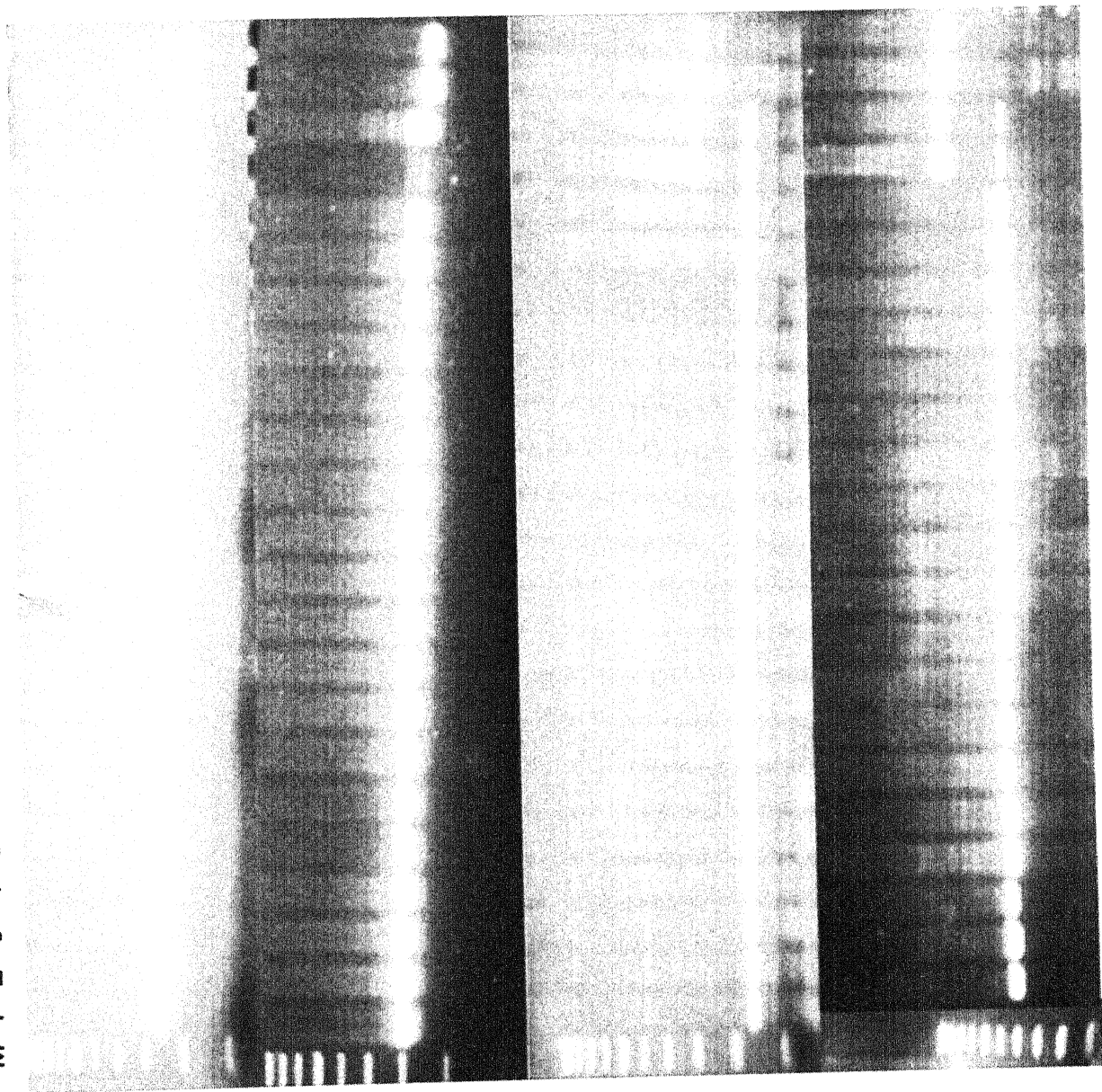


Fig 16B: PCR patterns with STS primer (A)EF3/ER3 (B)ALF2/ALR2 (C)AF11/AR1 (D)T3F3/T3R3 (top to bottom). Lanes 1-19 *S. seabrana*, Lanes 20-23 *S. viscosa*, M = 100 bp ladder

according to the sequences by Liu and Musial (1995) and Vander Stappen *et al.* (1999). The polymerase chain reaction (PCR) amplification for STS loci followed the procedure described by Liu *et al.* (1996) with modification in genomic DNA concentration and inclusion of primer extension step for 5 min as a last step of amplification. The total reaction volume was 25  $\mu$ l, which contained 78.2 mM Tris-HCL (pH 8.8), 19.4 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.53 % (v/v) Triton X-100, 233  $\mu$ g/ml BSA, 5.8 mM  $\text{MgCl}_2$ , 130 mM dNTPs, 0.3  $\mu$ M of primers, 1.2 unit of Taq DNA polymerase (Bangalore Genei, India) and 25 ng of genomic DNA templates. The reactions were overlaid with mineral oil. The PCR protocol was as follows: 32 cycles of 94  $^\circ\text{C}$ /60 s, 55  $^\circ\text{C}$ /60 s, 72  $^\circ\text{C}$ /90 s; one cycle at 72  $^\circ\text{C}$ /5 min; and 4  $^\circ\text{C}$  soak in an MJ Research PTC-200. Amplification products were separated on 1.6 % agarose gels in 0.5X TBE buffer and visualized by staining with ethidium bromide. STS primers also produced good results as RAPD, when 17 pairs of STS were employed with 23 accessions of *Stylosanthes* (Fig. 16 A and B). These primers sets are basically developed on the coding and noncoding part of the important gene (Table 11 ). Maximum sharing of bands was observed with *S. seabrana* and *S. scabra* and minimum was observed when three species were taken together. The proportion of STS bands sharing was also high in *S. seabrana* and *S. scabra*. The maximum number of bands (5) shared was observed with primer sets SsCS247P1/P2 between *S. scabra* and *S. seabrana* whereas primers pairs SsCS4P1/P2 and SsCS15P1/P2 showed sharing of 4 bands between *S. scabra* and *S. viscosa*. However, total number of bands shared by *S. scabra* and *S. seabrana* was high. The maximum number of bands observed was 17 with primer set SsCS15 P1/P2 and minimum 2 with primers SsCS284P1/P2 and SHCAPEF3/R3. The unique bands with respect to the species also varied among these species and maximum number of such markers were obtained with *S. seabrana*, however STS markers revealed similar level of species-specific bands in *S. seabrana* and *S. scabra*. Table shows the unique and rare bands as amplified with 17 STS markers. Out of 17 primers tested, 11 amplified unique species-specific bands, which range from 1 to 4 per primer sets. Apart from unique bands, rare bands were also counted which was present in two or more than two species, and it ranges from 1 to 9 in each accessions. In total 66 rare bands were observed. Since rare bands were those which have similar molecular weight in two or more than two species, the importance of these bands cannot

**Table 11.** List of STS primers tested, number of total number of bands amplified and sharing of bands among three species.

STS primer pairs	Sequences	Function	References	Intron	Target region	Total no. of bands amplified	% of polymorphism	Sharing of bands as revealed by STS primers		
								<i>S. scaberrana</i> <i>S. scabra</i>	<i>S. scabra</i> & <i>S. viscosa</i>	<i>S. scaberrana</i> & <i>S. scabra</i> & <i>S. viscosa</i>
SHST1F1	5' GAAGCAAACTCTCTTCACAT 3'	Sulfate transporter	Reddy et al., 1996	No	C	3	100	0	2	0
SHST1R1	5' GGCTTGTCAGGGAAGAAAGT 3'	Sulfate transporter	Reddy et al., 1996	-	NC	3	100	1	1	0
SHST1F3	5' GCAACTTATGTCCTCCAGAC 3'	Sulfate transporter	Reddy et al., 1996	-	NC	3	100	1	1	0
SHST1R3	5' TCACCAATCATGAAGCTG 3'	Sulfate transporter	Reddy et al., 1996	-	NC	3	100	1	1	0
SHCADI12	5' GCTTAGGCCATGTTGCCATC 3'	Cinnamyl alcohol dehydrogenase	Curtis et al., 1995	Yes	C	10	100	3	1	0
SHCADIAR2	5' ATCTCTGAGTCTCTCTTCAG 3'	Sulfate transporter	Reddy et al., 1996	Yes	C	3	100	1	0	0
SHST3F3	5' GGTTAAACATAATAAAGCATG 3'	Phenylalanine ammonia lyase	Smith et al., 1995	-	NC	3	100	1	0	0
SHST3R3	5' GTCTTGTAACAATTCACAGC 3'	Phenylalanine ammonia lyase	Smith et al., 1995	-	NC	3	100	1	0	0
SHPALF2	5' TTCACGGCAATGTGCCAAGG 3'	Phenylalanine ammonia lyase	Smith et al., 1995	-	NC	3	100	1	0	0
SHPALR2	5' AGGTATTGTATCTGTGCCA 3'	Phenylalanine ammonia lyase	Smith et al., 1995	-	NC	3	100	1	0	0
SHCAPEAF1	5' TAATGTTGTCTTGTGCTG 3'	Cationic peroxidase gDNA	Liu et al., 1996	Yes	C	5	100	1	1	0
SHCAPEAR1	5' GGTGCTCAAAAGCTGACAAAC 3'	Cationic peroxidase gDNA	Liu et al., 1996	Yes	C	5	100	1	1	0
SHCAPEF3	5' TCCAGTGGCCAGATTAGGAC 3'	Cationic peroxidase gDNA	Manners et al., 1995	-	NC	2	100	0	1	0
SHCAPER3	5' CCACACAGTTTATGAGAGG 3'	Cationic peroxidase gDNA	Reddy et al., 1996	Yes	C	12	91.66	2	1	1
SHST3F1	5' TAATCTTGGCCAGCTCA 3'	Sulfate transporter	Reddy et al., 1996	Yes	C	4	75	1	1	1
SHST3R18	5' CTGCACCTGCCATGAATCCAC 3'	Sulfate transporter	Reddy et al., 1996	Yes	C	4	75	1	1	1
SHST2F3	5' AAGAACAAAGAACTCTTCTGG 3'	Sulfate transporter	Reddy et al., 1996	Yes	C	4	75	1	1	1
SHST2R16	5' CCATGTAGTCACTGCTGACCGAG 3'	Sulfate transporter	Reddy et al., 1996	Yes	C	4	75	1	1	1
SHCAPEAF1	5' TAATGTTGTCTTGTGCTG 3'	-	Curtis et al., 1995	Yes	NC	6	83.33	1	1	1
SHCAPEAR12	5' TAGCCCACTCTGCGTCC 3'	-	Curtis et al., 1995	Yes	NC	6	83.33	1	1	1
SHCAPEAF11	5' GCTCAACACAAAGACCTTG 3'	-	Curtis et al., 1995	Yes	NC	3	100	1	1	0
SHCAPEAR1	5' GCTGCTCAAAAGCTGACAAAC 3'	-	Curtis et al., 1995	Yes	NC	3	100	1	1	0
SsCS247P1	5' CCAGATTGGTTCGGATTCC 3'	-	Liu and Musial, 1995	-	-	14	92.85	5	0	1
SsCS247P2	5' GAGAAACAGATGGCATCAGA 3'	-	Liu and Musial, 1995	-	-	3	66.66	0	2	1
SsCS268P1	5' CAGCGGTGGAGAAAGAAAG 3'	-	Liu and Musial, 1995	-	-	2	50	0	0	1
SsCS268P2	5' AGGAAACAGTCTGGAAGATAATG 3'	-	Liu and Musial, 1995	-	-	3	66.66	0	2	1
SsCS284P1	5' TCCGAAACACAGACACAGG 3'	-	Liu and Musial, 1995	-	-	2	50	0	0	1
SsCS284P2	5' AAGGTCTGCCATGGTATTGT 3'	-	Liu and Musial, 1995	-	-	11	63.63	0	4	4
SsCS4P1	5' ACAAGGTCCAAGAGACAAAC 3'	-	Liu and Musial, 1995	-	-	3	66.66	0	0	1
SsCS4P2	5' ACATTTCTTCTCCACAGC 3'	-	Liu and Musial, 1995	-	-	3	66.66	0	0	1
SsCS71P1	5' ATCTTCTCAATGCTTTTCGGA 3'	-	Liu and Musial, 1995	-	-	3	66.66	0	0	1
SsCS71P2	5' AGAAGCGCATGCAAGGAAATC 3'	-	Liu and Musial, 1995	-	-	17	94.11	2	1	1
SsCS15P1	5' GGTCCTCCATAGAACTGC 3'	-	Liu and Musial, 1995	-	-	17	94.11	2	1	1
SsCS15P2	5' GCTACTGGGCTTTTGCC 3'	-	Liu and Musial, 1995	-	-	17	94.11	2	1	1

be ignored. In case of rare bands the bands that is either present in two species *i.e.*, *S. scabra* and *S. viscosa* or in *S. scabra* and *S. seabrana* were 25 and 21 respectively indicated their role in future improvement of *S. scabra*. The natural occurrence of *S. scabra* has been largely to the region of origin of stylo *i.e.*, Brazil. The presence of many lines of *S. seabrana* in these regions, which is close to the vicinity of the origin of diversity, further, augmented the higher proportion of its genome in *S. scabra*.

In total 104 STS fragments from 17 primer pairs were used to generate tree of cluster analysis. Genetic relationship study using STS marker system further enhances the accuracy of relationship study among *Stylosanthes*. Dendrogram generated on the STS bands revealed two major clusters namely cluster I and cluster II having 29% genetic similarity level to each other (Fig. 16C). Cluster I was further subdivided into two subclusters namely subcluster I and subcluster II having genetic similarity of 59%. Accession CPI 110372 of subcluster I of cluster I was observed as a distinct node having 95% genetic similarity to the rest of the accessions of this subcluster. Cluster II embodied two accessions of *S. viscosa*, possessing 72% similarity to each other. Subcluster II of cluster I embodied two *S. scabra* accessions (RRR 94-97 and Seca) having 100% genetic similarity. Among nineteen accessions of *S. seabrana* IG 357, IG 391, IG 370, IG 325 and CPI 105546 B showed 100% similarity among them. Similarly, IG 355, CPI 2534, CPI 2539, IG 346, IG 339, IG 384, IG 352, EC 408403, EC 408404 and CPI 104710 showed 100% genetic similarity among them. Of the 19 accessions 16 showed 100% similarity among them but grouped at three places of cluster I. As in lane of RAPD, STS also showed CPI 110372 as a separate node. Two accessions of *S. scabra* are made a distinct cluster having similarity more than 95%. These two accessions joined with *S. seabrana* accession at the similarity level of 62%. *S. viscosa* accessions showed 71% similarity to each other (Table 12).

When two data sets were combined the resultant dendrogram was observed more similar to RAPD based dendrogram (Fig. 16D). Dendrogram revealed two major clusters namely cluster I and cluster II having 34% similarity to each other. Cluster I consisted nineteen accessions of *S. seabrana* and two accessions of *S. scabra*. Whereas cluster II



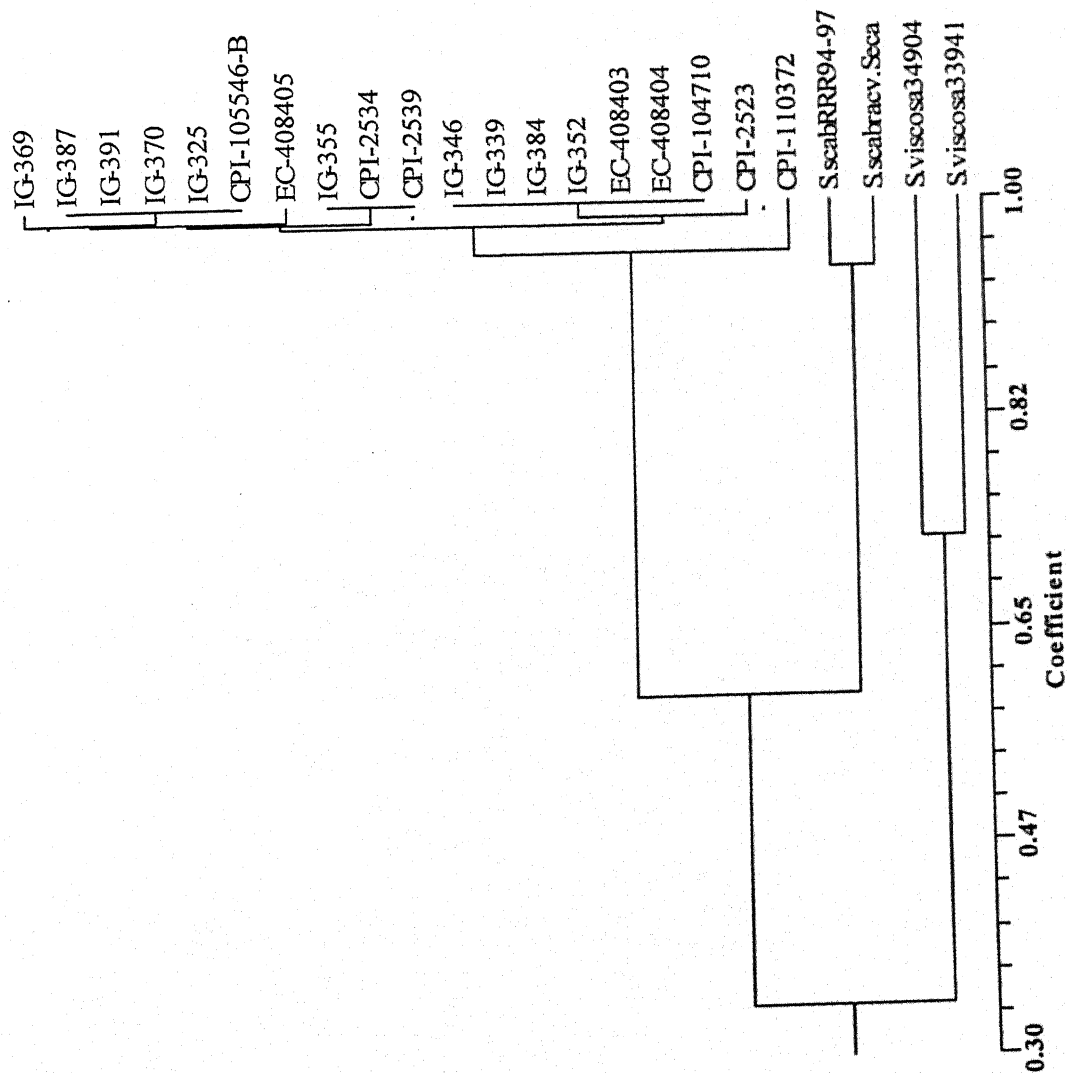


Fig 16C: Dendrogram showing genetic relationship among five species of *Stylosanthes* comprising 23 accessions as revealed by STS markers base on UPGMA of DICE similarity coefficients and SAHN clustering.

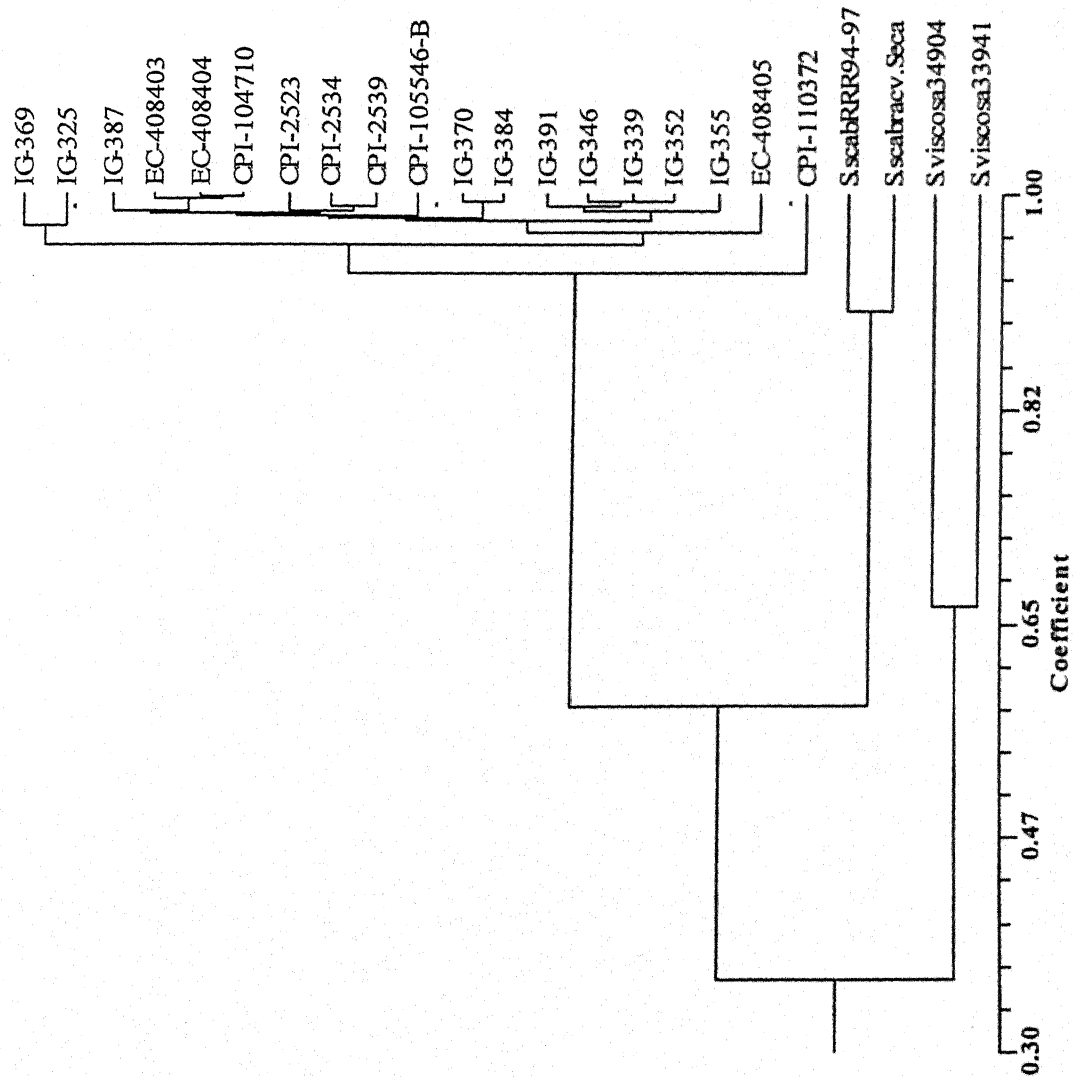


Fig 16D: Dendrogram showing genetic relationship among five species of *Stylosanthes* comprising 23 accessions as revealed by combining RAPD and STS markers base on UPGMA of DICE similarity coefficients and SAHN clustering.

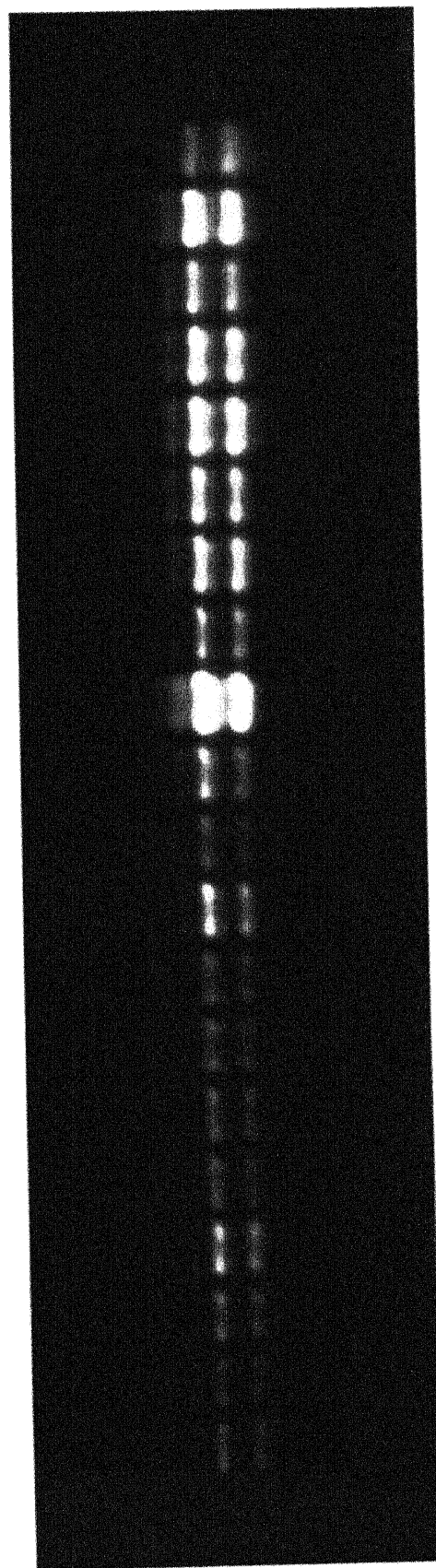
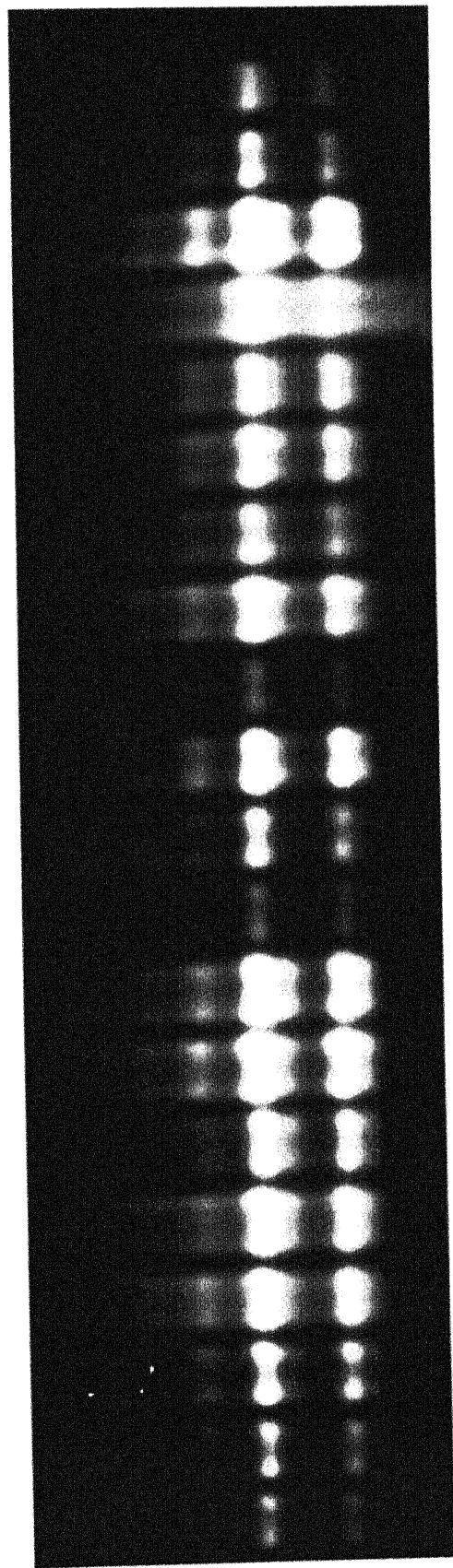


comprised two accessions of *S. viscosa*. This dendrogram pattern indicated more closeness of *S. seabrana* accession with *S. scabra*. Cluster I was divided into two subclusters namely subcluster I and subcluster II having 57% genetic similarity among them. Subcluster I of cluster I comprised of *S. seabrana*, while subcluster II possessed *S. scabra* accessions. In subcluster I IG 369, IG 325 EC 408405 and CPI 110372 accessions were more divergent to rest of the accessions of *S. seabrana* indicating variation among nineteen accessions of *S. seabrana*. EC 408403, EC408404 and CPI 104710 were 100% similar. Likewise IG 346, IG 339 and IG 355 were 100% similar. Subcluster II of cluster I comprised two accessions of *S. scabra* having 90% similarity among them. Cluster II consisted *S. viscosa* with 67% genetic similarity among them. Results showed that similarity between *S. scabra* accessions was more in comparison to two *S. viscosa* accessions (Table 13).

#### Gene expression by RT-PCR analysis

Total RNA was isolated from five species of *Stylosanthes* at different levels of salinity as well as at different stages of stress. RNA was isolated from 4-6 whole and fresh seedlings using Qiagen plant RNA isolation kit. Two thick bands of RNA was observed on 1.0% agarose gel (Fig. 17A). In total nine primers pairs (Table 11) were utilized for RT-PCR analysis. These primer pairs were synthesized, based on the gene sequence of sulfate transporter, cinnamyl alcohol dehydrogenase, phenylalanine ammonia lyase and cationic peroxidase gDNA. Of these nine primer pairs, five namely T3F1/T3R18, T2F3/T2R16, IAF2/IAR2, AF1/AR1 and EF3/ER3 reacted well with total RNA of five species of *Stylosanthes* (Fig 17B).

In case of *S. viscosa*, primer pairs T2F3/T2R16 and IAF2/IAR2 showed better response in terms of over expression of genes at different levels of salinity. With both primers, expression was maximum in control condition. Expression of genes, though less was also observed in plants exposed to different levels of salinity indicating down regulation of genes under increasing level of salinity. Primer pairs T3F1/T3R18 and AF1/AR1 have not shown significant expression of genes (Fig. 17C). In *S. humilis* overall expression of

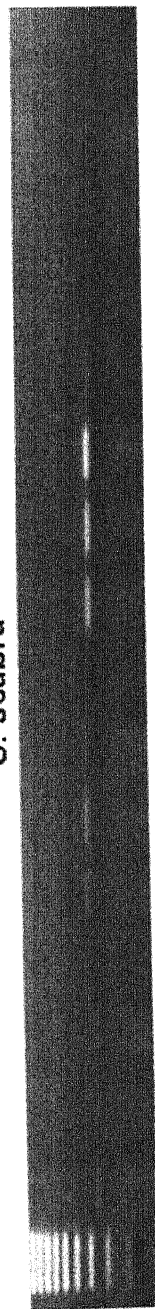


**Fig 17A: Total RNA isolated from control and salinity stressed seedlings of *Spilosanthus* using RNA isolation kit**

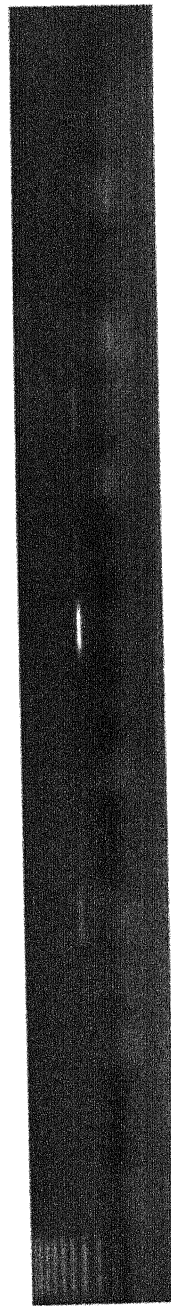
M C T1 T2 T3 C T1 T2 T3 C T1 T2 T3  
*S. seabraana*



M C T1 T2 T3 C T1 T2 T3 C T1 T2 T3  
*S. scabra*



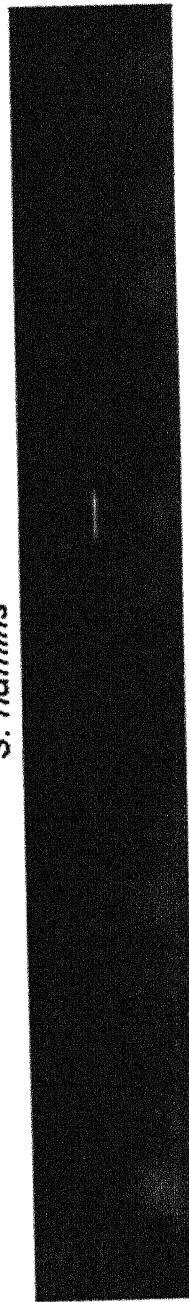
M C T1 T2 T3 C T1 T2 T3 C T1 T2 T3  
*S. viscosa*



M C T1 T2 T3 C T1 T2 T3 C T1 T2 T3  
*S. hamata*



M C T1 T2 T3 C T1 T2 T3 C T1 T2 T3  
*S. humilis*



**Fig 17B RTPCR analysis with four primer pairs namely T3F1/T3R18, T2F3/T2R16, IAF2/IAF2 and AF1/AR1 RNA isolated after 3 days of exposure of 50, 100 and 150mM of NaCl**

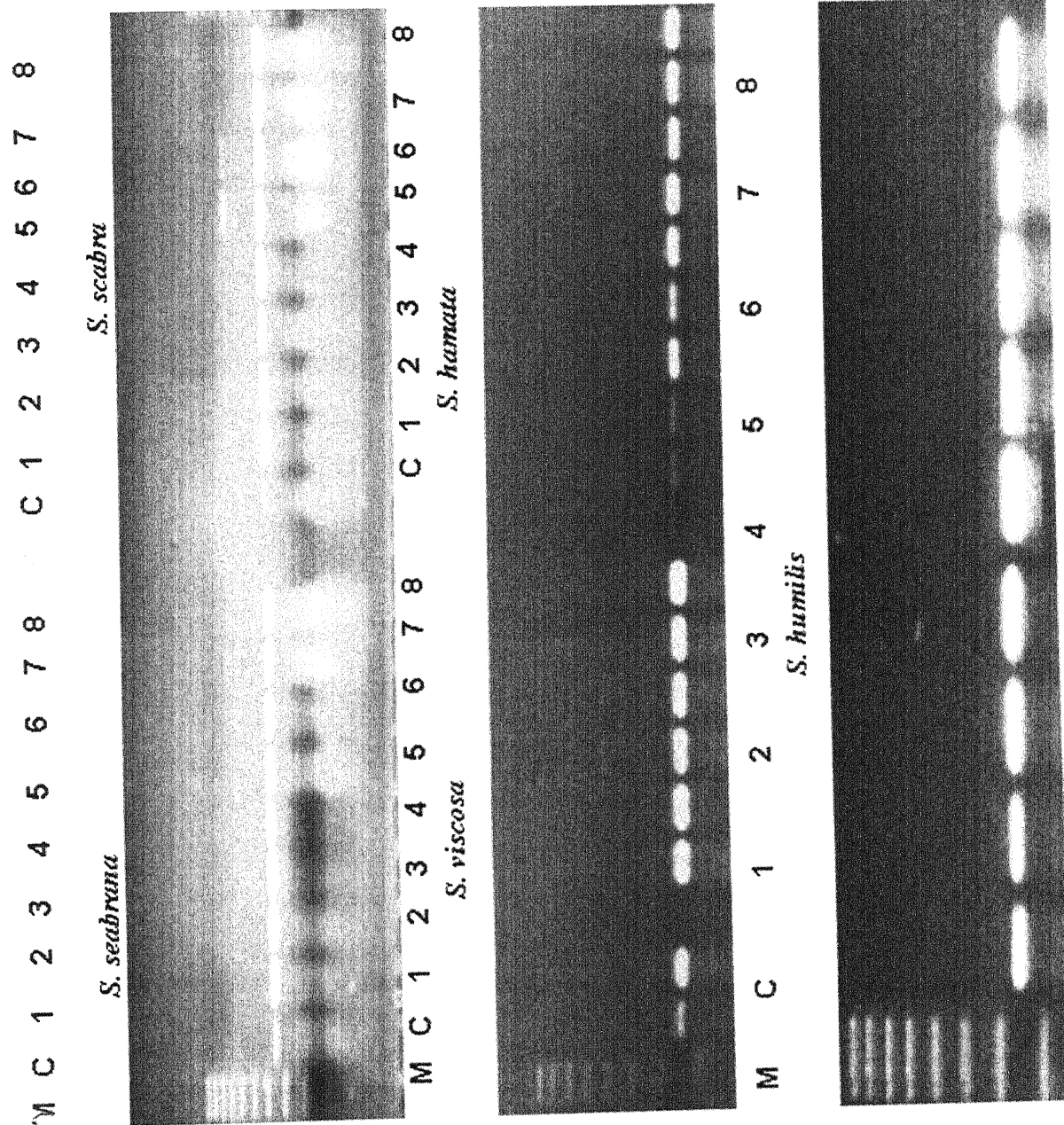


Fig 17C: RT PCR analysis using primer pairs SHCADI2/SHCADI2R in five species of stylosanthes. C = control, Lanes 1,3,5,7 contains RNA from 75mM stressed seedlings isolated at 2,6,24 and 48 hrs of stress,2,4,6, 8 contains RNA from 150mM stressed seedlings isolated at 2, 6, 24 and 48 hrs of stress

all four genes was low, however the expression with primer pairs T3F1/T3R18, T2F3/T2R16, IAF2/IAR2 was in order of increasing with the increasing level of salinity indicating up regulation of genes in this species. Expression of genes corresponding to primer pair EF3/ER3 i.e., cationic peroxidase was also not observed in *S. humilis*. In case of *S. hamata*, expression of genes was observed more at 50mM of NaCl stress with all four primer pairs. With increased stress, expression was observed down regulated with T3F1/T3R18, T2F3/T2R16 and AF1/AR1 primer pairs, however with primer pair IAF2/IAR2 expression of gene was higher with increasing stress. Higher expression of genes was observed with *S. seabrana*, specifically at higher level of NaCl stress. Primer pairs T2F3/T2R16, IAF2/IAR and AF1/AR1 indicated up regulation of gene up to 100mM stress. Further increase in NaCl stress, gene expression was observed down regulated. With primer pair T3F1/T3R18 expression of genes was less. Up-regulation of gene expression was observed in *S. scabra* with primer pairs T3F1/T3R18, T2F3/T2R16 and IAF2/IAR2 with increasing level of salinity. Primer pair AF1/AR1 has not shown much gene expression in *S. scabra* when stress was imposed.

At 100mM of NaCl stress, RNA was isolated at 2, 6 and 24 hours of stress and gene expression was studied using four primer pairs namely T3F1/T3R18, T2F3/T2R16, IAF2/IAR2 and AF1/AR1. Primer pairs T2F3/T2R16 and IAF2/IAR2 showed well expression of genes. Expression of genes with primer pair IAF2/IAR2 was better than those with primer pair T2F3/T2R16 with the increasing duration of salt stress. In *S. seabrana* both primer pairs indicated up-regulation of gene expression at 100mM NaCl stress. The level of expression was more in control as well as at 6 hour of NaCl stress (Fig. 17 D). Expression of genes was highest at 24 hour of stress. *S. scabra* also showed maximum expression at 24 hour of stress. In general up regulation of gene expression was observed with increasing duration of salt stress. Gene expression in *S. viscosa* corresponding to T2F3/T2R16 and IAF2/IAR2 primer pairs was also up-regulated with increased duration of salt stress. In *S. viscosa*, expression with primer pair IAF2/IAR2 was much higher than those with primer pair T2F3/T2R16. In *S. hamata* also expression of genes was up regulated with increasing duration of salt stress. Specifically the expression was more in control as well as at 6 hour of NaCl stress. At 24 hour of stress, *S.*

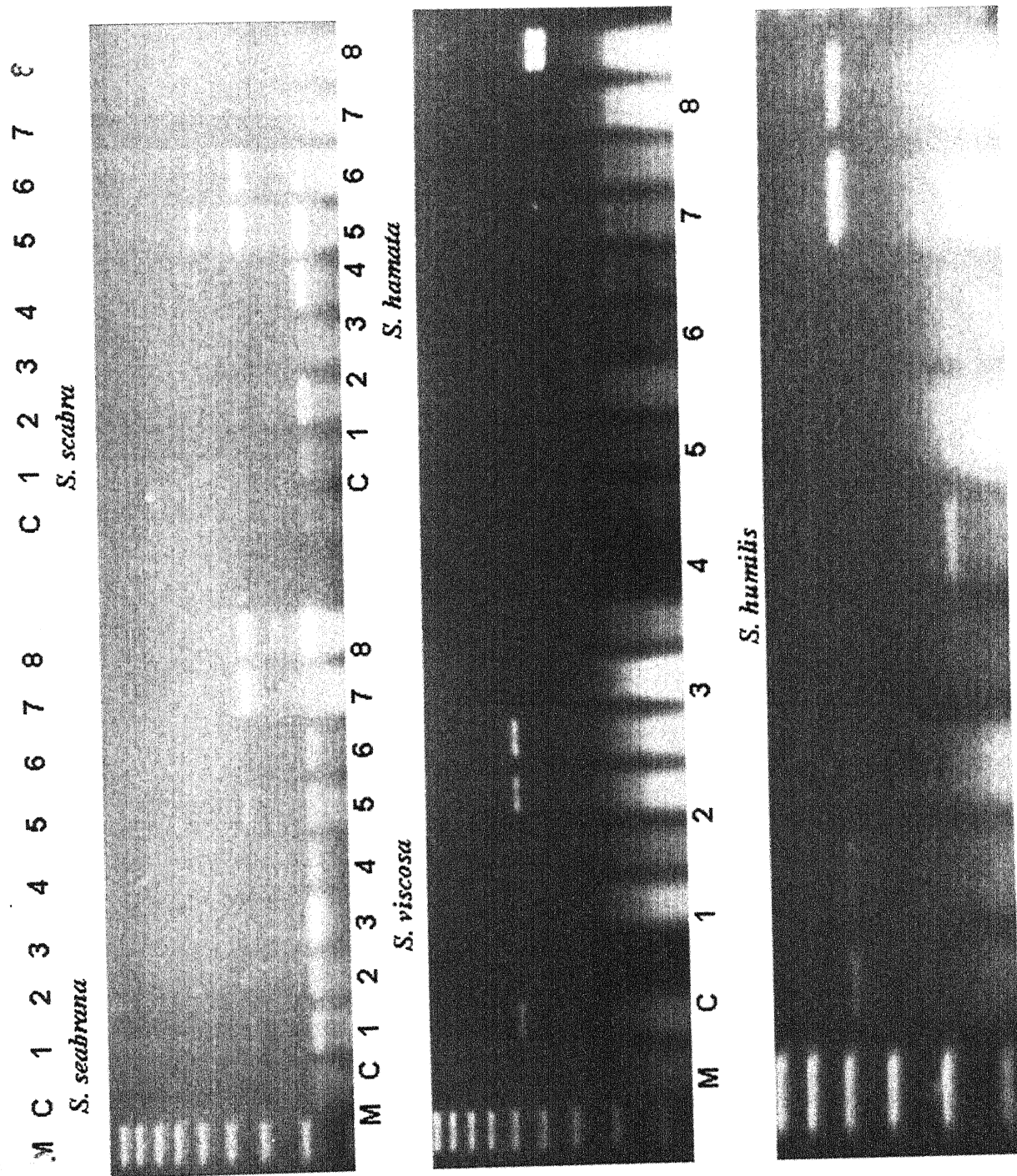


Fig 17D: RT PCR analysis using primer pairs SHCAPEAF1/SHCAPEAR1 in five species of stylosanthes. C = control, Lanes 1,3,5,7 contains RNA from 75mM stressed seedlings isolated at 2,6,24 and 48 hrs of stress, 2,4,6, 8 contains RNA from 150mM stressed seedlings isolated at 2, 6, 24 and 48 hrs of stress

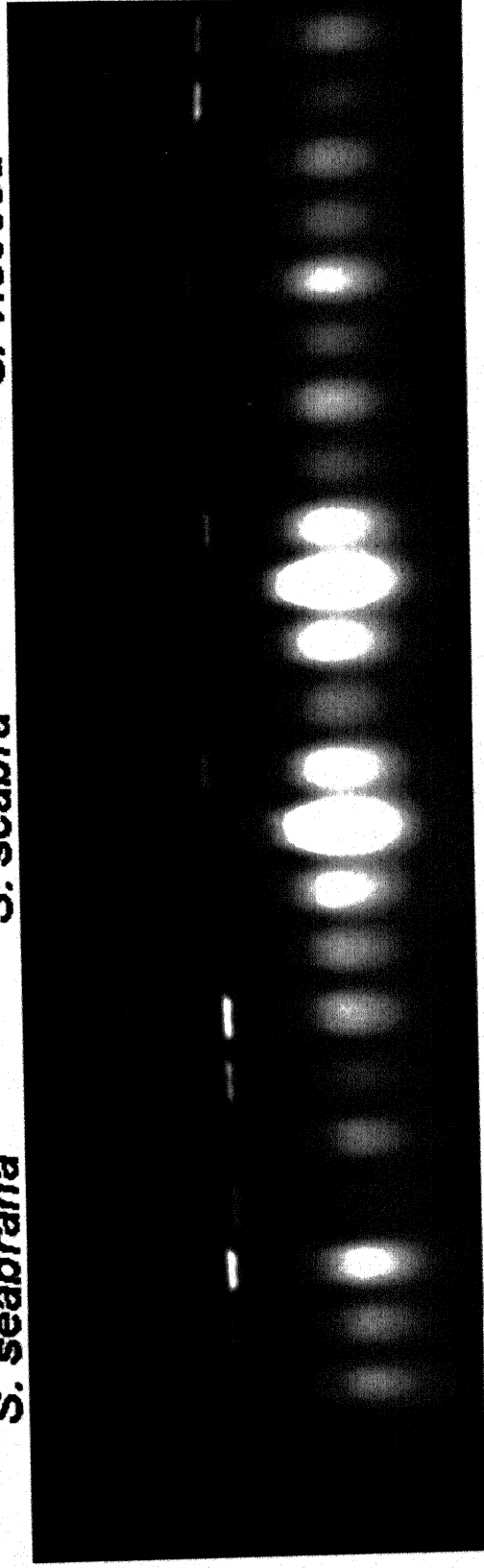
*hamata* showed less expression of genes. Up-regulation of gene expression was observed in *S. humilis* with increasing duration of salt stress and expression was more at 6 hour of NaCl stress.

Total RNA was also isolated at two different (75mM and 150mM) NaCl salinity at 2, 6, 24 and 48 hour of stress and gene expression study was performed with primer pairs T2F3/T2R16, IAF2/IAR2, AF1/AR1 and EF3/ER3. In *S. seabrana*, expression of genes with primer pair T2F3/T2R16 was less in control. At both stress levels genes expression was high till 24 hour and afterwards expression declined. *S. scabra* also showed less expression in control. Up-regulation of gene expression was observed at both stress levels with increasing stress duration. Gene expression was observed significant in *S. scabra* with the increasing exposure of salt stress. Expression of genes in control of *S. viscosa* was also less. Up-regulation of gene was observed in *S. viscosa* with both stress levels. In *S. hamata* expression of genes in control was much less as compared to other species. At 75mM of NaCl stress, down regulation of gene expression was observed whereas at 150mM expression was more after 2 and 6 hour of stress whereas it declined at 24 and 48 hour. *S. humilis* showed less expression of genes in control. At both stress levels i.e., 75 and 150mM gene expression was up regulated till 24 hour and afterwards gene expression declined. With primer pair EF3/ER3, *S. seabrana*, *S. hamata* and *S. humilis* showed lower gene expression as compared with *S. scabra* and *S. viscosa* (Fig 17F-H). Less expression of genes was also observed in *S. seabrana* at 24 hour of stress in both stress levels, whereas *S. hamata* showed no gene expression with both stress levels as well as with increased duration of salt stress. *S. humilis* showed gene expression only at 150mM of stress and that to at 48 hour of stress duration. Up-regulation of gene expression was observed in *S. scabra* at both stress levels as well as with increased duration of stress. Less gene expression was shown by *S. scabra* in control. Expression was up-regulated in *S. viscosa*. In control, gene expression was well observed. At 75mM and 150mM stress levels genes were highly expressed up to 48 hours of stress. With primer pair IAF2/IAR2 all species showed better gene expression at 75mM and 150mM with increasing duration of salt stress. In *S. seabrana* gene expression was less in control. Gene expression was up regulated in both stress levels as well as with increased salt

*S. viscosa*

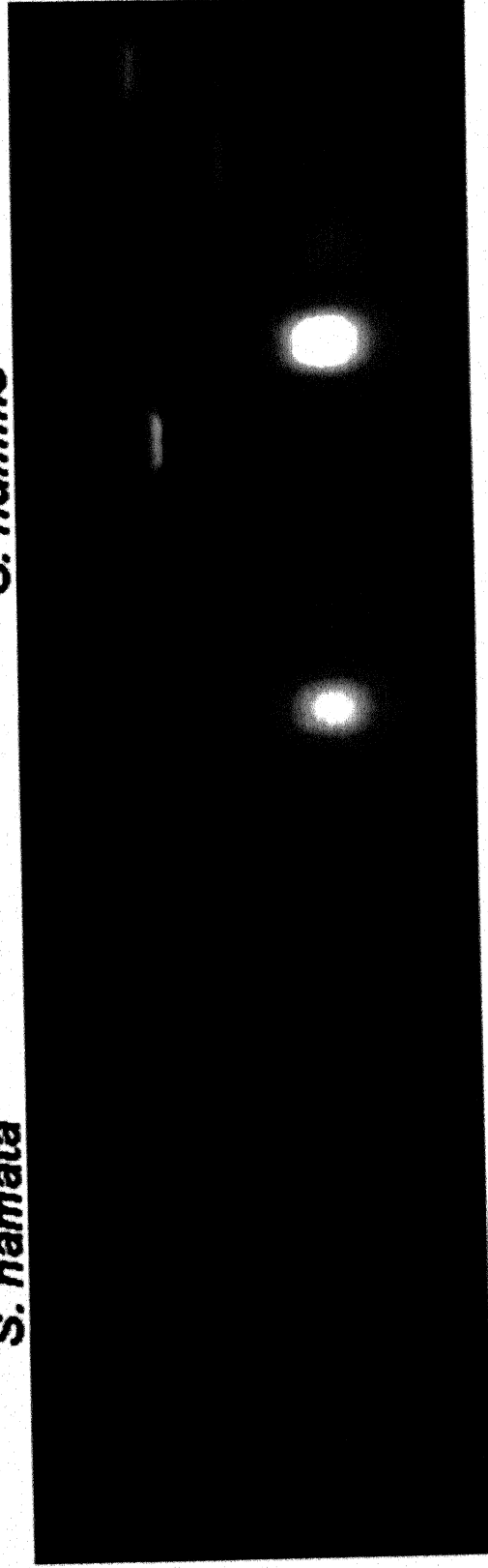
*S. scabra*

*S. seabrana*

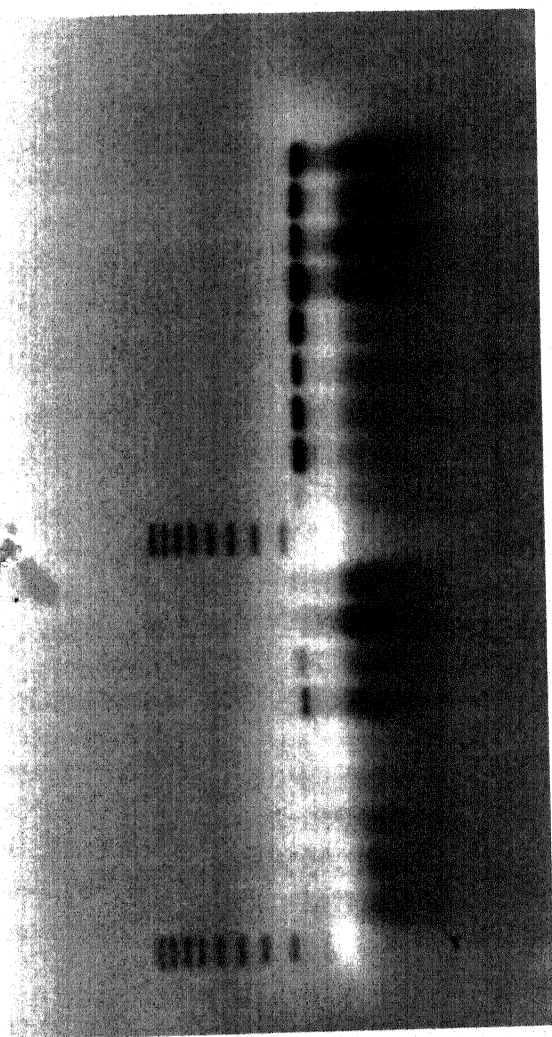


*S. humilis*

*S. hamata*

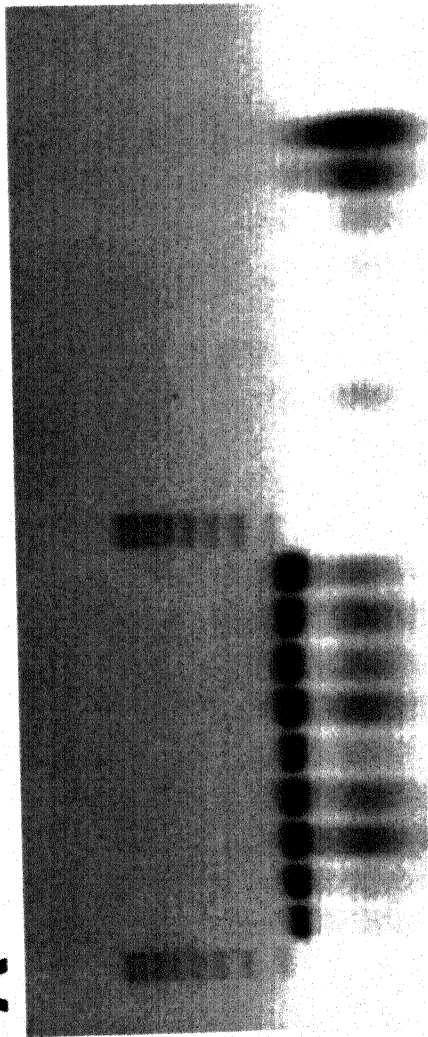


**Fig 17E: RT PCR analysis after 3 days exposure of salt (C, 50, 100 and 150mM) with two sets of primers T2F3/T2R16, IAF2/IAR2**



**A**

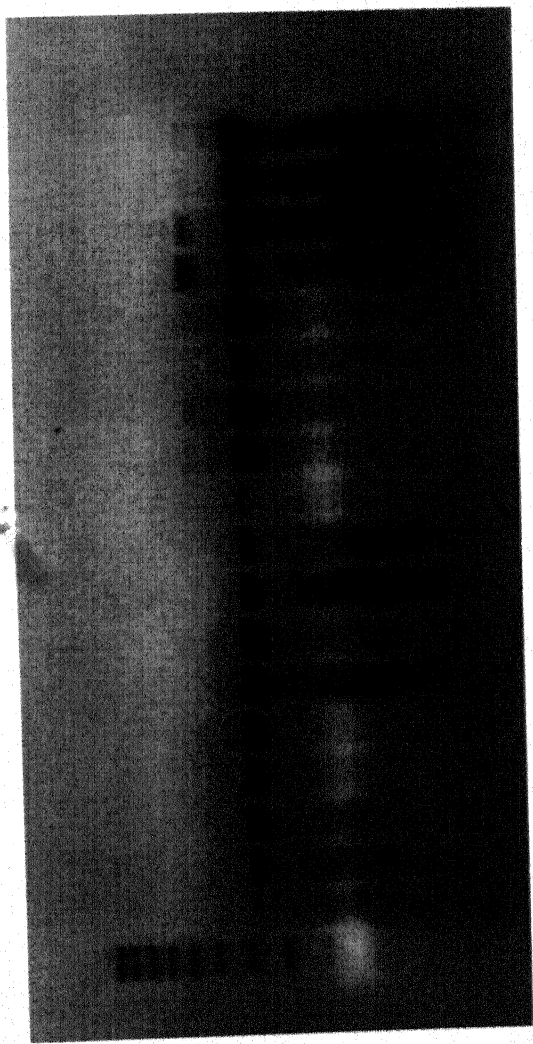
**B**



**C**

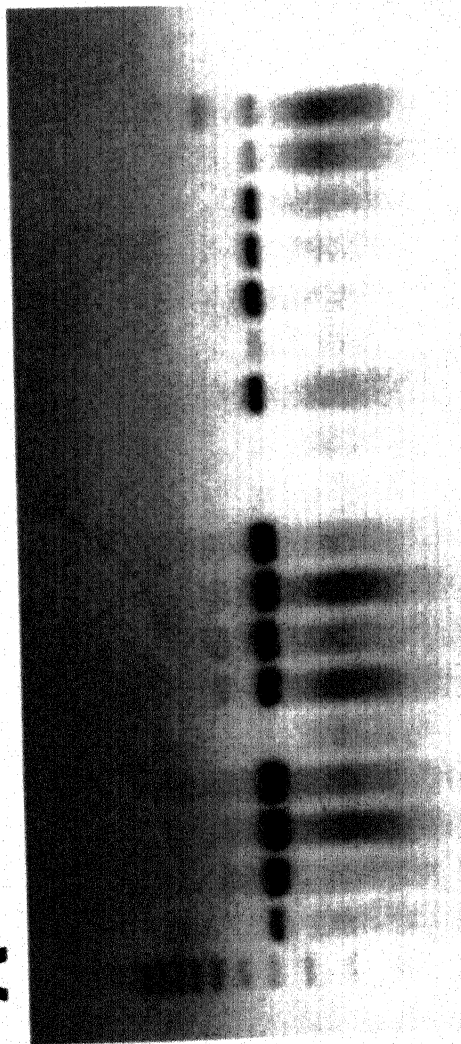
**D**

**Fig17F. RT-PCR of five species of stylo with STS primer pair EF3/ER3.**  
**A. *S. scabrana*, B. *S. scabrana*, C. *S. viscosa*, D. *S. hamata*.**



**A**

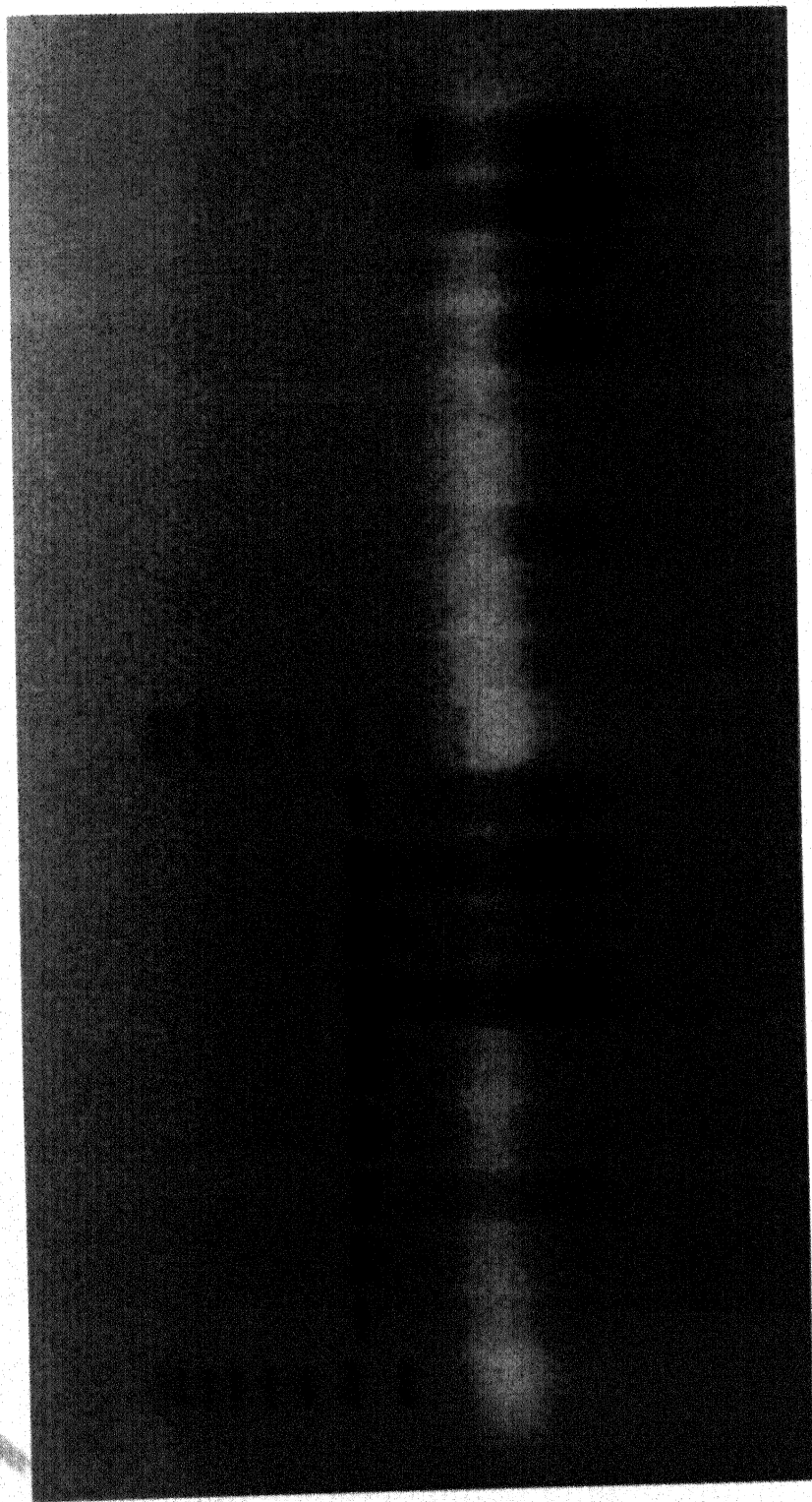
**B**



**C**

**D**

Fig 17G. RT-PCR of four species of stylo with STS primer pair T2F3/T2R16 at 2, 6, 24 and 48 hours with 75mM and 150mM of NaCl stress.  
A. *S. scabrana*, B. *S. scabrana*, C. *S. viscosa*, D. *S. hamata*.



**B**

**A**

**Fig 17H. RT-PCR of *S. humilis* with STS primer pair**

**A. T2F3/T2R16, B. EF3/ER3 at 2, 6, 24 and 48 hours with 75mM and 150mM NaCl stress.**

duration. *S. scabra* also showed less expression in control, whereas with both stress levels expression of genes was high with increased salt duration. Though in control of *S. viscosa*, less expression of genes was observed but it was more than those of other species in control. *S. hamata* showed less expression of genes in comparison to other species. At 75mM of NaCl stress gene expression was observed up regulated with increased duration of salt stress. At 150mM of salt stress expression of genes was more than 75mM of stress, and was up regulated with increasing exposure of salt stress.

Primer pair EF3/ER3 showed low gene expression with increased duration of salt exposure in all species of *Stylosanthes*. In control all species showed less gene expression, whereas gene expression was up regulated in *S. seabrana* at both stress levels i.e., 75mM and 150mM. With increased duration of salt stress gene expression was enhanced at 6 hour in 75mM and in 48 hour at 150mM of stress. *S. scabra* also showed up regulation of gene expression with increased exposure of salt stress at both stress levels. In *S. viscosa* primer pair EF3/ER3 showed no gene expression with the salt exposure. *S. hamata* showed up regulation of gene expression with increased exposure of salt stress in both stress levels. Expression was high in 24 and 48 hours of stress. In *S. humilis*, genes expression was more at 6 hour and 24 hours in both levels of stress corresponding to the stress stages and in both stress levels.

## DISCUSSION

The genus *Stylosanthes* (Fabaceae) constitutes more than forty species (Kirkbide and De Kirkbide, 1985). Five species namely *S. seabrana*, *S. hamata*, *S. guianensis*, *S. viscosa* and *S. scabra* are predominantly used as fodder legume in humid to semi-arid tropics of the country (Chandra *et al.*, 2006; Ramesh *et al.*, 1997). Due to its ability to restore soil fertility, improve soil physical properties and provide permanent vegetation cover it is playing a vital role in the development of wastelands in India. However, the most important limitation attached with this crop is the narrow genetic variability and low availability of diverse accessions. *Stylosanthes* is regarded as the most important range legume for the humid to semi-arid tropics. It is extensively utilized in pastoral, agropastoral and silvipastoral systems for animal production. It is also considered as nurse crop in plantation on degraded lands. Large gap in requirement and availability of forage seeds is a major constraint in development of fodder resources in the country. The importance of livestock in Indian agriculture is well recognized. However, low productivity of livestock is a matter of concern, which is mainly due to the poor fodder and feed resources. With the increasing livestock population in India it becomes imperative to produce more forage biomass to meet the demand. There exist a critical shortage of fodder i.e. 80 million tones dry and 660 million tones green as reported by Hazra (1995). To overcome this situation, there is need to increase fodder production by efficient utilization of all the land resources and technologies.

Nearly 10% of the total land surface is covered with different types of salt affected soils. No continent in our planet is free from salt affected soils. They are distributed not only in desert and semi desert regions, but also frequently occur in fertile alluvial plains, river valleys and coastal areas close to densely populated areas and irrigation systems. Salt affected soils usually have low biological activity both because of osmotic and ionic effects of salts and due to limitation of carbonaceous substrates. Microbial growth is depressed in sodic (alkali) soils due to, at least in part, limitation in carbon substrate (carbon stress) and in saline soils due to salt stress. Saline soils are those that develop under the influence of electrolytes of sodium salts with nearly neutral

reaction (dominantly NaCl, Na<sub>2</sub>SO<sub>4</sub> seldom NaNO<sub>3</sub>). These soils occur mainly in arid and semiarid regions and form a major part of all the salt affected soils of the world. High contents of soluble salts accumulated in these soils can significantly decrease their value and productivity. In saline soils it is the high salt concentration in the solid and liquid phases which results in high osmotic pressure, hindering the normal development of plants. The stress factor is the salinity with all its disadvantages consequences of plant life. Apart from this, some compounds of the salt content of these soils, for example, chlorides as toxic elements, also act as one of the stress factors. Besides the salt affected soils developing as a result of natural soil forming processes, the so called secondary salt affected soils have an increasing importance that is both scientific and practical. Secondary salt affected soils are those which have been salinized owing to manmade factors, mainly as a consequence of improper methods of irrigation. The extension of secondary salt affected soils is rather sizeable, and this adverse process is as old as irrigated agriculture itself. The process of salinization is also advancing vigorously at present, and more than half of all the irrigated lands in the world are under the influence of secondary salinization. When speaking of the manmade factors of salinization, we also have to mention potential salt-affected soils which are not salt-affected at present, but in case of the extension of irrigation, deforestation, overgrazing and other manmade measures, can will be salinized unless the necessary preventive procedures are undertaken in due time. No global records are available of the size of potential salt-affected soils; however, the area that they cover is larger than that of existing salt-affected soils. Secondary salt-affected soils can be divided into the following two categories: secondary formation of salt affected soils caused by irrigation and secondary formation of salt-affected soils caused by human activities other than irrigation.

#### **Germination study under salinity:**

The work embodied in the present thesis is to evaluation and identify the suitable species of *stylo* that will grow well under salinity condition. The lines identified are also molecularly characterized to estimate the level of genetic relationships among species /accessions. The isoform which are qualitatively and quantitatively showed differences is also identified would be suitable for identification of salinity tolerance lines. Gene

expression analysis through RT-PCR is also performed to further forfeit the data obtained at biochemical level.

Germination is a complex phenomenon involving many physiological and biochemical changes leading to the activation of embryo. However, during initial phase of germination any unfavorable change may jeopardize the process of germination to a large extent. Salinity, as an abiotic hazard, induces numerous disorders in seeds during germination. It either completely inhibits germination at higher levels or induces a state of dormancy at lower levels. It first reduces imbibition of water because of the lowered osmotic potential of the medium. Second, it causes toxicity; that is, it changes enzyme activity, hampers protein metabolism, upsets plant growth regulators balance, and reduces the utilization of seed reserves. It may elicit changes at ultrastructural, cellular and tissue or even at organ levels. Salinity interacts with certain plant and environmental factors during germination. Among the plant factors, seed coat, dormancy, seed age, seed polymorphism and most important seedling vigor are prominent. Germination of a viable seed starts with the imbibition of water and terminates with emergence of embryonic tissues. This involves the hydration of proteins, subcellular structural changes, respiration synthesis of macromolecules, and cell elongation. Growth of embryonic tissues as an important step in the completion of germination, establishment of seedlings under stressful conditions is also important. Establishment of seedlings seems crucial under salinity, as without a successful crop stand simple emergence of embryonic tissues will prove futile. The process of germination is greatly influenced by the nature and extent of salinity and, above all, on the behavior of seeds. During study of germination vigor in *stylo*, *S. seabrana*, *S. hamata* and *S. humilis* showed almost similar patterns of germination while *S. scabra* and *S. viscosa* showed retardation of germination. Germination started from proper imbibition of seeds to the fresh young seedlings. However, *S. scabra* and *S. viscosa* performed late on seed imbibition, and they late germination. Germination percentage of these two species was also very low as compared to *S. hamata*, *S. seabrana* and *S. humilis* under salt conditions. Length of seedlings was higher in *S. hamata*, *S. humilis* and *S. seabrana* and they exposed healthy as compared to the seedlings of *S. scabra* and *S. viscosa*. Seedlings of *S. hamata*, *S. humilis* and *S.*

*seabrana* were well grown up to the 150mM NaCl concentration. But in *S. scabra* and *S. viscosa*, imbibition as well as germination vigor was low. Reduced hydration of embryo and cotyledon and reduced water uptake was reported when NaCl was used as a growing medium (Poljakoff - Mayber *et al.*, 1994; Wahid *et al.*, 1998; Katerji *et al.*, 1994; Petruzzeli *et al.*, 1991). Salt stress hampered the metabolism of stored materials and growth of the embryo. At the onset of germination, synthesis of enzymes and changes in the metabolic pattern were initiated, but salt stress either alters it or does not permit the synthesis of specific metabolites required for germination. Application of salinity hampers the utilization and mobilization of materials required for the production of seedlings by affecting the enzymatic activities of seed essential for these reactions.

Seed coat was the first barrier to the entry of water and ions into the seed. The hard and thick seed coat offered resistance to the entry of water into the seed and minimized the contact of ions with the embryo as in case of seeds of *Stylosanthes*. Size of seeds was also a major factor in germination under stress. In *S. hamata* and *S. humilis* seed size was large, perhaps it was a reason for better salt tolerance of these two species. Thick seed coat also acts as a buffering agent to ionic toxicity (Poljakoff *et al.*, 1994; Eshie, 1995) and enhances germination under the influence of salt toxicity at seedling stage.

#### **Protein content and isozyme profile**

Salinity creates major impact on the activities of the enzymes for protein metabolism. Except, *S. scabra* all species selected in the present study showed increase in protein content under stress imposition. Their protein synthesis was not much affected by salinity at third day of salt stress as was in *S. scabra*. *S. scabra* showed no change in protein content to the 100mM salt stress, but at 150mM, content of protein decreased and again at 200mM it increased. At fifth day of stress, major decrease in protein content was observed in *S. scabra*. At third and fifth day of salt stress *S. hamata*, *S. seabrana*, *S. humilis* and *S. viscosa* maintained higher protein level at 100mM stress, but at higher or 200mM, decrease in protein content was observed in all four species. Ramgopal, 1990 reported that the synthesis of eight new proteins in germinating barley embryo under salt

stress and seven during recovery (Aquilla *et al.*, 1993; Khan *et al.*, 1989). Maximum increase in total soluble proteins under stress in *S. hamata* over other species indicated that this particular species showed better ability to tolerate salinity in comparison to other species. To maintain the osmotic level as it has been suggested that in general increase in amino acid level is one of the major attributes of stress to maintain the osmotic level (Good and Zaplachinski, 1994). Most amino acids concentration increased during the induction of stress indicated that two species of *Brassica* showed better stress response, however the level of methionine did not increase under such conditions. Better performing lines of rice also showed higher level of protein under stress (Reddy *et al.*, 1998). Many researchers have reported overall changes in protein synthesis with the onset of stress. Native protein study of five species of *Stylosanthes* under salt stress indicated that during stress condition new bands of proteins were emerged to overcome salt stress. In the present study it has been observed that the level of distinct polypeptide increased and some of the polypeptides decreased. The intensity of many bands increased under stress indicated higher accumulation or synthesis of proteins during stress. As the number of bands varied among the species the over expression of protein also varied indicating different levels of tolerance against stress present in the species. As it has been reported that the level of proteins maintains the osmotic level of plants during stress. The induction of many polypeptides under stress as evident from protein analysis indicated importance of the proteins in providing the stability of the cell and cell's environment. In isozyme study, all the fourteen isozymes reported quantitative change in protein bands under salt stress conditions. Results also showed that under stress new isoforms of stress proteins were formed in some of the enzyme systems. All the isozyme systems provided discriminatory tool for evaluating their response to salinity stress. Few bands like ACP-6 and 9 in *S. seabrana*, ACP-4 and 10 in *S. hamata*, EST 13 in *S. humilis* disappeared. Newly appeared bands were like EST-4 and 5 in *S. seabrana* and *S. scabra* respectively. SOD-2 in *S. viscosa*, MDH-2 in *S. humilis* appeared as new bands, under salinity as compared to control. The study indicated that several genes were involved in governing salt toxicity tolerance whereas few remained unaltered across the stress gradient, and may be used as biochemical marker for selection of tolerant species against salt toxicity. At higher levels of stress, both decrease and increase in intensity of many enzymes were

observed. This indicates gradual degradation of these enzymes or their structural modification under increased salt toxicity levels. The banding patterns expressing differential intensity, showed the varying status of an enzyme affected by the stress. Such isozyme bands can be considered as "injury markers". Roy and Mandal (2005) studied modulation in isozyme profiles induced by Fe-toxicity tolerance in rice. Disappearance of some bands at higher levels of stress indicated that isoforms become unstable under excess NaCl stress. Swapna, (2003) also indicated the induction of enzyme activities under different levels of NaCl stress in rice. Few isoforms disappeared at varying levels of stress, although appeared at low stress level (50mM) like SOD 5 in *S. scabra*, EST 2 in *S. humilis* but disappeared at higher stress levels. These results showed that some isozymes could be degraded under different levels irrespective of increment of NaCl concentration. Some bands like EST 1 and EST 10 in *S. scabra*, EST 7 in *S. viscosa*, SOD 1 and 3 in *S. seabrana*, ACP 1 in *S. scabra*, *S. viscosa* and *S. humilis* remain unaltered across the stress gradient suggesting their stability in stress environment. These stable bands of isozymes belonging to esterase, superoxide dismutase, acid phosphatase, malate dehydrogenase and peroxidase enzyme systems may be used as biochemical markers in selection of tolerant plants against NaCl toxicity. There was also found increase in activity of proteins that largely required for stress conditions during germination of seedlings.

#### **Malondialdehyde content**

Salt stress induced production of free radicals caused lipid peroxidation and membrane deterioration in plants. The level of this deterioration was determined by measuring the level of malondialdehyde (MDA) contents. In the present study the level of MDA content increased at 50mM NaCl in *S. hamata*. However, with the increase in salinity stress (100mM) magnitude of MDA content decreased but further increased at 150mM salt stress. In *S. seabrana*, *S. scabra*, *S. viscosa* and *S. humilis*, MDA content increased narrowly at 50mM, however major increase was noticed at 100mM and 150mM. The difference in MDA content has been reported in two species of jute and thus the difference in membrane permeability as a result of increased peroxidation (Choudhury and Choudhury, 1985). Sudden increase of MDA with salt stress caused higher lipid

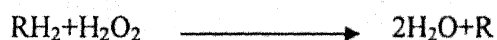
peroxidation and thus the species would be more vulnerable to stress. The level of MDA sharply increased in stress indicating higher lipid peroxidation (Dhindsa and Matowe, 1981). The decrease in lipid peroxidation (MDA content) has been attributed to the expression of genes, Glutathione synthetase (GS) and sulfur metabolism (HAC2) causes increase in glutathione, an indicator of oxidative stress resistance in transgenic lines of tobacco (Singh and Verma, 2001). Similarly calli of some selected lines of rice showed less accumulation of MDA and were found superior to other lines in terms of stress tolerance (Reddy *et al.*, 1988).

### **Proline content**

The level of proline increased with magnitude of stress. Seedlings of *Brassica juncea*, *Sesbania sesban* and *Oryza sativa* when exposed to 200mM NaCl, they have accumulated about 10, 20 and 40 fold higher levels of proline than controls (Shiv kumar *et al.*, 1998). Seedlings of *Stylosanthes* showed increased proline content up to 100mM NaCl however at 150mM proline content decreased at third day of stress. Upon increasing the stress duration proline content increased with increasing level of salinity. Seedlings of *S. hamata* showed highest increase in proline content in salt stress duration. In the sand experiment, leaves of *Stylosanthes* showed increased proline content at first week of experiment but at harvesting stage it was decreased in all the species indicated early response to salinity stress in leaves. In general excessive accumulation of proline in plants under stress is considered to be an adaptive mechanism to allow essential metabolic events to continue normally. Proline has been shown to play a vital role in maintaining the membrane integrity and in stabilization of macromolecules or molecular assemblies (Schwab *et al.*, 1990; Genard *et al.*, 1991). Proline is one of a member of organic solutes that accumulate in a wide range of organisms, from bacteria to higher plants, exposed to environmental stresses, drought, salinity, heavy metals, low or high temperature. This accumulation increased the resistance to the various stresses (Aspinall and Paleg, 1981).

### Change in enzyme activities caused by salt stress:

Under high salinity decrease in total glutathione and an increase in total ascorbate accompanied with enhanced glutathione reductase and ascorbate peroxidase activities were observed in leaves. Chaparzadeh (2004) investigated marigold (*Calendula officinalis* L.) under long term salt induced oxidative stress at 0, 50 and 100 mM NaCl. Decrease in superoxide dismutase and peroxidase also induced by salinity. The decrease in dehydroascorbate reductase and monodehydroascorbate reductase activities suggests that other mechanisms play a major role in the regeneration of reduced ascorbate. The changes in catalase activities, both in roots and in leaves, may be important in  $H_2O_2$  homeostasis. Though the level of increase in peroxidase (POD) activity and also the days on which maximum activity attained by a particular species varied among the species. In general response of salt stress observed was the increase in peroxidase activity measured using guaiacol as an artificial substrate. Peroxidase catalyzes hydrogen peroxide-dependent oxidation of substrate ( $RH_2$ ) according to the general equation:



Many researchers have reported increase in peroxidase activity under salt (Siegel, 1993). It indicates the formation of large amounts of  $H_2O_2$  during salt stress. Elevated  $H_2O_2$  concentrations could release peroxidase from membrane structures.

Increasing behavior in peroxidase, catalase, ascorbate peroxidase, glycolate oxidase and glutathione reductase activity in seedlings and leaves of *Stylosanthes* showed that salt stress could increase the accumulation of peroxidase substrates, such as glutathione, ascorbate and phenolic compounds, which in turn, are scavengers of activated oxygen species. Similar observations have been reported by Elstner (1982), Winston (1990). Guaiacol peroxidase may not be as important in scavenging  $H_2O_2$  as CAT and enzyme in the ascorbate-glutathione cycle because the peroxidase determined with the guaiacol belongs to the first group (Asada, 1992) and its main function is lignification rather than detoxifying the free radicals (Zhang and Kirkham, 1994). Peroxidases usually divided into two groups. The first group of peroxidases render physiological function (lignification) whereas the second group consists of enzymes

whose primary function is  $H_2O_2$  scavenging includes glutathione reductase, ascorbate peroxidase and glycolate oxidase.

Superoxide dismutases are metallo-proteins catalyzes dismutation of the superoxide free radical ( $O_2^{\cdot -}$ ) to molecular oxygen and  $H_2O_2$ . Seedlings of *Stylosanthes* showed first increase in SOD at lower stress level of 50mM and then decreased activity at 100mM. It was increased again at 150mM and at higher stress level of 200mM, SOD activity decreased. Maximum increase in activity was shown by *S. hamata* and *S. humilis*. Minimum change in activity was reported in *S. scabra* and *S. viscosa*. Leaves of five species of *Stylosanthes* showed constant level of SOD activity with increasing level of salinity. It did not show any change in SOD activity. Greater activity of SOD has been reported in selected superior lines of rice showing tolerance to stress (Reddy *et al.*, 1998). The depression of SOD activity in plants reported in many crops (Choudhury and Choudhury, 1985). As SOD catalyzes the dismutation of superoxide anion radical ( $O_2^{\cdot -}$ ) with great efficiency, resulting in the production of  $H_2O_2$  and  $O_2$  (Smirnoff, 1993; Winston, 1990), the decrease in SOD activity could impair the  $O_2^{\cdot -}$  scavenging system of cells and favor accumulation of  $O_2^{\cdot -}$  which mainly contribute in damaging the membrane. Because measured enzyme activity is a result of both synthesis and degradation, any decrease in net SOD activity under salinity can be ascribed to either reduced synthesis or enhanced degradation of the enzyme. In addition, accumulation of  $H_2O_2$  under salinity also could lower SOD activity provides more supporting references.

#### Genetic relationships study in *Stylosanthes*

Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the effective and efficient utilization of plant genetic resource collections. Among the several markers available the isozyme, random amplified polymorphic DNA (RAPD) and sequence tagged sites (STS) data can be generated faster and with less labour than other methods, such as RFLP (restriction fragment length polymorphism) and micro satellites. However, isozyme studies are limited by the numbers of enzymes and loci that can be resolved, and reveal only genetic changes in coding regions of the genome that have resulted in an altered amino acid

sequence. Additionally, lack of allozyme polymorphism may further restrict its application. In contrast RAPD analysis enables the detection of informative genetic markers at a large number of loci in both coding and noncoding regions of the genome (Williams et al., 1990). Stappen (1999) and Liu (2004) reported 19 STS primer pairs on coding and non coding regions based on published *Stylosanthes* genes, which were mostly derived from cDNA. Liu *et al.*, (1996) tested 20 STS markers to determine genetic relationships among 63 genotypes representing 24 *Stylosanthes* accessions. Total 148 alleles were amplified and analyzed, resulting in a genetic similarity value ranging from 0.62 to 0.98 among the accessions. Cluster analysis revealed three main groups and three subgroups, and most of the species were classified unambiguously. Allopolyploid species were recognized by the occurrence of more than one allele per STS marker, indicating fixed heterozygosity. Of the twenty, sixteen STS markers were observed useful for the identification of genotypes within a species. Inter-species relationships, as revealed by STS, were in general agreement with the previously reported morphological and molecular relationship studies. These STS markers were useful as an additional tool for the identification of species, sub-species and genotypes in *Stylosanthes*, with a view to plant conservation and breeding. Curtis *et al.*, (1995) analyzed RFLP, using peroxidase *O*-methyltransferase, phenylalanine ammonia-lyase and coniferyl alcohol dehydrogenase cDNAs isolated from *Stylosanthes humilis*, as probes, provided molecular evidence for the genetic origin of the naturally occurring allotetraploid genotype *Stylosanthes hamata* cv. Verano ( $2n=4x=40$ ). Hybridization patterns strongly suggest that the likely progenitors of *S. hamata* cv. Verano were a diploid *S. humilis* ( $2n=2x=20$ ) and a diploid *S. hamata* ( $2n=2x=20$ ). Genetic contribution of two progenitors of *S. scabra* investigated in the present study employing RAPD and STS markers as earlier reports have conclusively indicated *S. seabrana* and *S. viscosa* as two diploid progenitors of allotetraploid *S. scabra* (Liu *et al.*, (2004). A total of 476 RAPD bands generated from 32 primers, 104 STS fragments from 17 primer pairs indicated significant relationship of *S. scabra* with that of *S. seabrana* in comparison to its other progenitor *i.e.*, *S. viscosa*. As revealed from dendrogram analysis indicated impact of close proximity of the occurrence of *S. seabrana* in the development of *S. scabra*. The proportion of bands shared by RAPD (40 %) and STS (40 %) markers were also observed high between *S. seabrana* and *S.*

*scabra*. However, the numbers of unique bands were less in all these species. More correspondence and sharing of bands by *S. seabrana* towards *S. scabra* have suggested using selected lines of *S. seabrana* in artificial synthesis of allotetraploid, drought tolerant *S. scabra*. Liu (1997) selected one hundred accessions of *S. scabra*, majority was collected from Brazil and most of the others came from either Colombia or Venezuela, to represent the geographical distribution of the *S. scabra* at Australian Tropical Forages Genetic Resource Centre, were analyzed using RAPD as markers. Seven of these accessions were found not to be *S. scabra*. Of the *S. scabra* accessions, the average dissimilarity value among Brazilian accessions (0.053) was much lower than that among Colombian (0.074) or Venezuelan (0.088) accessions, with an overall dissimilarity value of 0.059 among all the *S. scabra* accessions.

Dendrogram analysis of *Stylosanthes* derived from among nineteen accessions of *S. seabrana* IG 357, IG 391, IG 370, IG 325 and CPI 105546 B showed 100% similarity among them. Similarly, IG 355, CPI 2534, CPI 2539, IG 346, IG 339, IG 384, IG 352, EC 408403, EC 408404 and CPI 104710 showed 100% genetic similarity among them. Of the 19 accessions 16 showed 100% similarity among them but grouped at three places of cluster I. As in lane of RAPD, STS also showed CPI 110372 as a separate node. Dendrogram developed on RAPD, STS and combined data of bands showed *S. scabra* accessions are more closer to each other than *S. viscosa* accessions. Among nineteen *S. seabrana* accessions IG 369, IG 325, CPI 110372 were more divergent to rest of the accessions of *S. seabrana* indicating variation in *S. seabrana*.

#### Gene expression under salinity

Total RNA was isolated from five species of *Stylosanthes* at different levels of salinity as well as at different stages of stress. RNA isolated from 4-5 fresh seedlings yielded two thick bands of RNA on 1.0% TBE gel indicated good quality of RNA for RT-PCR analysis. In total nine primers pairs (Table) were utilized for RT-PCR analysis. *S. hamata* showed up-regulation of gene expression with increased exposure of salt stress in two stress levels. Expression was higher in 24 and 48 hours of stress. In *S. humilis*, gene expression was more at 6 hour and 24 hours in both levels of stress and that corresponds

to the stress stages at both stress levels. In control, all species showed less gene expression, whereas gene expression was up-regulated in *S. seabrana*, *S. hamata* and *S. humilis* at both stress levels i.e., 75mM and 150mM. With increased duration of salt stress gene expression was enhanced at 6 hour in 75mM and in 48 hour at 150mM of stress. *S. scabra* also showed up regulation of gene expression with increased exposure of salt stress at both stress levels indicated up-regulation of gene under salinity in majority of *stylo* species. In *S. viscosa* primer pair EF3/ER3 showed no gene expression with the salt exposure. However, with primer pair EF3/ER3, *S. seabrana*, *S. hamata* and *S. humilis* showed lower gene expression as compared with *S. scabra* and *S. viscosa*. Primer pairs that reacted well with *S. scabra*, *S. viscosa* and showed lower expression level with *S. hamata*, *S. humilis* and *S. seabrana* species will provide better insight in salt tolerance studies, and also can be used to differentiate among salt tolerant and susceptible lines. Rampino *et al.*, 2006 studied *Triticum* and *Aegilops* seedlings differing in their response to drought stress at the physiological and molecular levels. Modification in the expression level, of five dehydrin (DHN) genes have been reported utilizing reverse transcription-polymerase chain reaction (RT-PCR) and indicated a relation between the expression of these genes and tissue water content. In particular, in the resistant genotypes the expression of DHN genes was initiated even though tissue hydration levels were still high, indicating also in wheat the involvement of these proteins in water retention.

#### **Salinity stress and plant response**

Salinity is the most important environmental factor that cause osmotic stress and reduction in plant growth crop productivity. As a counter action of these effects, the water potential of the cells may be decreased by the synthesis and accumulation of osmolytes such as proline allowing additional water to be taken up. Osmotic adjustment has been widely proposed as a plant attribute that confers adaptation to abiotic stress as reported by Basnayake (1995). Osmolyte content was observed increasing with increasing concentration of NaCl. Apart from isozyme analysis and enzyme activities, water potential of *stylo* seedlings was uniformly decreased (more negative) with increasing magnitude of salt stress. Initially minimum water potential was observed in *S. humilis*. It also showed maximum change or decrease from control to stress stages.

During salt stress proline content increased gradually up to 100mM stress in *Stylosanthes* seedlings while at 150mM it decreased. Maximum proline content at higher stress level was observed in *S. hamata*.

The accumulation of compatible solutes may help to maintain the relatively high water content necessary for growth and cellular functions. Increase in proline content was high enough to be considered as principal solute in osmoprotection. Increase in proline content under salt stress was also reported by Mattioni (1997). Change in gene expression occurs under salinity. Gene expression study under salinity revealed that in control seedlings, expression was high due to high metabolism activity required for germination. During germination metabolic machinery was highly active to provide necessary products required for the seedling germination and their proper establishment in adverse environment. Genes encoding enzymes involved in the biosynthesis of proline, osmolytes and different enzymes required in osmotic recovery were encoded by enhance gene expression in response to salt stress. High expression of *S. seabrana*, *S. hamata*, *S. viscosa* and *S. humilis* with four STS primer pairs showed these species better over *S. scabra* under salinity.

## SUMMARY

Salinity is a widespread phenomenon and is estimated to affect 10% of the world's land surface. The worst affected regions are in the arid and semi-arid zones where population increase is greatest and food production is most vulnerable because of problems with over grazing, over cultivation and the large seasonal variability in rainfall. Salinity usually occurs in conjugation with other soil problems. In India saline soil is found in Rajasthan, Punjab, Haryana, Maharashtra, Madhya Pradesh, Uttar Pradesh, Orissa Andhra Pradesh and Tamil Nadu. The rate at which the salinity problem is increasing, there is a need to identify salt tolerant crops of economical importance. Looking into the increasing demands for fodder under problematic areas in order to compete country's needs there is an urgent need to identify suitable forages for such zones. *Stylosanthes* which is highly drought tolerant range legume, breeding programs utilizing suitable lines/species of stylo can be attempted as better forage for salinity areas even where water is a limiting factor. The present study is initiated with seven *Stylosanthes* species. Techniques used for selection procedure were screening under *in-vitro*, mixed salt, single salt, petri plate with different levels of NaCl and sand culture. The five selected species *S. seabrana*, *S. scabra*, *S. viscosa*, *S. hamata* and *S. humilis* were further biochemically and molecularly analyzed. All experiments were carried out at Indian Grassland and Fodder Research Institute's laboratory and glass house. The main emphasis was given on the following points.

1. Screening of *Stylosanthes* lines at different levels of salinity in different growing medium.
2. Monitor the biochemical changes including antioxidant activities in stress and un-stress plants.
3. Estimation of water potential, osmolality, proline and MDA content.
4. Genetic relationship study of selected lines showing different responses towards salinity.
5. Expression analysis of stress responsive genes in selected lines by doing RT-PCR.

The imbibition time of stylo seeds was observed better in *S. hamata* and *S. humilis* over *S. seabrana*, *S. scabra* and *S. viscosa*. Seed size of *S. hamata* and *S. humilis* was also big to grow more vigorously under salinity conditions. Maximum seed germination vigour and higher seedling length also placed these two species under the category of tolerant species against salinity. Small seed size, late imbibition, germination vigor, short seedling size lead to conclusion that *S. scabra* and *S. viscosa* lines were susceptible under salinity. *S. seabrana* was observed as moderate salinity tolerant species.

Experiments carried out at field by imposing two months of stress showed change in morphological attributes in stress over control. Plants height declined significantly in all species under stress. Study also indicated a significant reduction in both fresh and dry weight under stress. Fresh weight and dry weight of leaves of five selected species decreased linearly with increasing salinity concentration in secondary salinization experiment in sand. Yellowing of leaves was observed in *S. hamata* and *S. humilis*, however this may also due to annual nature of these two species. *S. humilis* shed their leaves under high salinity. Level of chlorophyll a, Chlorophyll b and carotene was decreased initially but increased significantly in higher level of salt stress of 150mM. Height of plants was also reduced in *S. seabrana*. Reduced leaf area and branching was observed in all five species.

Osmolality content was observed initially lower in *S. viscosa*, whereas higher in *S. hamata*, *S. scabra*, *S. seabrana* and *S. humilis*. Change in osmolality was observed minimum in *S. viscosa*, while *S. scabra* and *S. seabrana* showed similar level of change under salinity. Maximum increase in osmolality was observed in *S. humilis* and *S. hamata*. Water potential was uniformly decreased (more negative) with increasing magnitude of salt stress. Initially minimum water potential was observed in *S. humilis*. It also showed maximum change (decrease) from control to stress stages. At lower NaCl stress of 50mM, water potential is decreased whereas at higher stress levels it increased. *S. hamata* showed less water potential change over stress. Highest decrease in water potential was measured in *S. scabra*. However, in *S. seabrana* water potential was lesser than *S. hamata* under salinity.

The level of malondialdehyde, which generally described the extent of lipid peroxidation, altered with the duration of stress. Increased MDA content was observed in *S. hamata*, *S. scabra* and *S. humilis* at lower level of NaCl, whereas an initial decrease in its content was observed in *S. seabrana*. Decrease in MDA content was noticed as salinity increased in all species. Increase in NaCl level, induced increase in MDA content of *S. viscosa*, *S. hamata* and *S. humilis*. Decrease in MDA content was observed on 3<sup>rd</sup> day of salinity stress in *S. seabrana* and *S. scabra*. Highest MDA content was observed in *S. hamata* and *S. humilis* at 3<sup>rd</sup> day of stress. The difference in the level of MDA may be seen as the difference in the membrane permeability. Based on this, species namely *S. hamata* and *S. humilis* have been identified better tolerant and possessing better ability to withstand against stress.

The level of proline in leaves increased instantaneously when salt stress was imposed in pots. The increase in the level of proline may be because of the increase in the activity of a rate limiting enzyme, pyrroline-5-carboxylate synthetase. Possibly the increase in level of proline in pot stressed plants is because of the breakdown of protein occurred at the highest level of salt stress. Change in proline content was similar in *S. seabrana* and *S. scabra*. Maximum increase in proline content was observed in *S. hamata*, *S. viscosa* and *S. humilis*. At initial level of stress proline content increased and then decreased at higher stress level of 150mM. The increase in proline content can be attributed in some extent to the degradation of the protein.

Total soluble proteins measured at different days of salt stress indicated increase in its content with increasing magnitude of stress. Maximum increase in protein was observed in *S. hamata* and *S. humilis*. *S. scabra* showed minimum increase in protein. *S. seabrana* and *S. viscosa* showed moderate increase in protein.

The activity of enzymes namely catalase, peroxidase, glutathione oxidase, glycolate peroxidase and superoxide dismutase indicated that antioxidant system plays an important role in providing the stress tolerance in *Stylosanthes* as in other crops.

However, decreased behavior of the enzyme activities at 50mM NaCl and further increase in it at 100mM indicated their adaptability towards salinity at third day of stress in all species. Salt stress study in *Stylosanthes* species demonstrated that besides peroxidase all other antioxidant enzymes plays significant role in salt stress. Enzymes like catalase, SOD, glycolate oxidase and ascorbate-glutathione cycle were important in scavenging free radicals and as the activity of these enzymes increased at fifth day of stress indicated their role in salinity tolerance. Taken together the behaviour of enzymes, the differing activity patterns revealed by different species indicated that of the five species, *S. hamata* and *S. humilis* were better performing species under stress over other species.

As reported in many crops, change of the relative composition of several isozymes of SOD, esterase and peroxidase during stress, the disappearance and increase in polymorphism of polyphenol oxidase, amylase, GOT, CAT, malate dehydrogenase, peroxidase were also observed in *Stylosanthes* species investigated. Prominent difference includes changes in banding patterns/intensity, mobility and number of polymorphic loci with respect to salt toxicity. Bands like ACP-6 and 9 in *S. seabrana*, ACP-4 and 10 in *S. hamata*, EST 13 in *S. humilis* disappeared and EST-4 and 5 in *S. seabrana* and *S. scabra* respectively, SOD-2 in *S. viscosa*, MDH-2 in *S. humilis* appeared as new isoform in the salinity condition as compared to control. The study indicated that several genes were involved in governing salt toxicity tolerance whereas few remained unaltered across the stress gradient, and may be used as biochemical marker for selection of tolerant species against salt toxicity. At higher levels of stress, both decrease and increase in intensity of many enzymes were observed. This indicates gradual degradation of these enzymes or their structural modification under increased salt toxicity levels. The banding patterns expressing differential intensities, showed the varying status of an enzyme affected by the stress. Such isozyme bands can be considered as "injury markers". Disappearance of some bands at higher levels of stress, indicate that isoforms become unstable under excess NaCl stress. The protein profile indicated absence of some polypeptides as well as appearance of fresh polypeptides under extreme stress and also the re-synthesis of some polypeptides in higher stress stages.

RT-PCR analysis showed that in control expression was low but when the stress was imposed expression was increased greatly. Increased expression was observed in all species. As the duration of stress was increased expression was increased. Some primer pairs were reacted well with *S. seabrana*, *S. hamata* and *S. humilis* but they show no reaction with *S. scabra* and *S. viscosa*. These primers can be used to differentiate between tolerant and susceptible species of *Stylosanthes*. Expression study by RT-PCR of seedlings at 2, 6, 24 and 48 hour with 75 and 150mM of NaCl stress revealed that expression was higher in both stress levels at 24 and 48 hour of stress. at 150mM stress expression was high from initial stress duration of 2 hour as compared with 75mM of stress in all species.

Dendrogram developed on RAPD and STS bands showed *S. scabra* accessions were more closer to each other as *S. viscosa* accessions. Among nineteen *S. seabrana* accessions IG 369, IG 325, CPI 110372 were more divergent to rest of the accessions of *S. seabrana* indicating variations among in *S. seabrana* accessions. As in case of RAPD, STS also showed CPI 110372 as a separate node. Among nineteen accessions of *S. seabrana* IG 357, IG 391, IG 370, IG 325 and CPI 105546 B showed 100% similarity among them. Similarly, IG 355, CPI 2534, CPI 2539, IG 346, IG 339, IG 384, IG 352, EC 408403, EC 408404 and CPI 104710 showed 100% genetic similarity among them. Some banding patterns indicated that of the 19 accessions, 16 showed 100% similarity among them but grouped at three places in cluster I. when RAPD and STS data sets were combined and dendrogram developed, the clustering of different accessions of *S. seabrana*, *S. scabra* and *S. viscosa* were almost same as obtained separately. The boot strap analysis carried out with STS data sets indicated (> 50 boot strap values) significant clustering of many accessions.

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